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(54) **Title:** METHODS AND COMPOUNDS FOR INHIBITING CELL PROLIFERATIVE DISORDERS CHARACTERIZED BY ABNORMAL *abl* ACTIVITY

(57) **Abstract**

The present invention concerns methods and compounds for inhibiting cell proliferative disorders characterized by abnormal *abl* activity. The preferred compounds described herein inhibit cell proliferative disorders by targeting abnormal *abl* activity. The preferred target is abnormal *abl* autokinase activity.

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DESCRIPTION

Methods and Compounds for Inhibiting Cell Proliferative Disorders Characterized by Abnormal *abl* Activity

Related Applications

The present application claims priority from an Israeli application, Application No. 105707 and is a continuation-in-part of "METHODS AND COMPOUNDS FOR INHIBITING CELL PROLIFERATIVE DISORDERS CHARACTERIZED BY ABNORMAL *abl* ACTIVITY" filed April 27, 1994, [Serial Number to be assigned]; and "METHODS AND COMPOUNDS FOR INHIBITING CELL PROLIFERATIVE DISORDERS CHARACTERIZED BY ABNORMAL *abl* ACTIVITY" filed April 28, 1994, [Serial Number to be assigned]; the entire contents of these prior applications are incorporated by reference into the present application.

Field of Invention

The present invention concerns methods and compounds for inhibiting cell proliferative disorders characterized by abnormal *abl* activity. Examples of cell proliferative disorders characterized by abnormal *abl* activity include forms of leukemia such as chronic myelogenous leukemia and acute lymphoblastic leukemia.

Background

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. These processes include, but are not limited to, cell proliferation, differentiation and survival. A central feature of signal transduction is the reversible phosphorylation of certain proteins. (For reviews, see Posada, J. and Cooper, J.A., 1992, *Mol. Biol. Cell* 3:583-392; Hardie, D.G., 1990, *Symp. Soc. Exp. Biol.* 44:241-255). The phosphorylation state of a protein is modified through the reciprocal actions of tyrosine

kinases (TKs) which function to phosphorylate proteins, and tyrosine phosphatases (TPs) which function to dephosphorylate proteins. Normal cellular function requires a delicate balance between the activities of these two types of enzymes.

The family of tyrosine kinases can be further subdivided into receptor-type and cytoplasmic proteins. The intracellular, cytoplasmic tyrosine kinases may be broadly defined as those protein tyrosine kinases which do not contain a hydrophobic, transmembrane domain. Bolen, *Oncogene* 8:2025-2031, 1993, reports that 24 individual tyrosine kinases comprising eight different subfamilies of cytoplasmic tyrosine kinases have been identified. All of the cytoplasmic tyrosine kinases are thought to be involved in signaling pathways that modulate growth and differentiation.

The Philadelphia chromosome has been associated with cell proliferative disorders such as chronic leukemia and acute lymphoblastic leukemia (Pendergast *et al., Cell* 75:175-185 (1993)). The Philadelphia chromosome (PH^+) was found in over 90% of human chronic myelogenous leukemia patients, and in a much smaller percentage of acute lymphoblastic leukemia patients (Ramakrishnan and Rosenberg, *Biochimica et Biophysica Acta* 989:209-224, (1989)).

The Philadelphia chromosome results from a reciprocal translocation between chromosomes 9 and 22 (Ramakrishnan and Rosenberg *supra*). In this translocation the *c-abl* gene, located on chromosome 9q, is translocated into chromosome 22, within the *bcr* gene, resulting in the formation of a chimeric *bcr-abl* gene encoding a *bcr-abl* fusion protein. Different types of *bcr-abl* fusion protein can result from a Philadelphia chromosome such as fusion proteins p185 *bcr-abl* and p210 *bcr-abl* weight. The p210 contains 937 bcr-encoded residues; the p185 form appears to share the first 455 amino acids with p210.

The K562 cell line, originally established from a patient with chronic myelogenous leukemia in the terminal blast crisis stage, can be induced to erythroid differentiation by reducing the level of p210 *bcr-abl* by specific antisense oligonucleotides, or inhibiting its tyrosine kinase activity by general tyrosine kinase blockers such as herbimycin A, genistein and erbstatin (Szczlik C, *et al.*, *Science 253*:562 (1991) and Honma Y, *et al.*, *Cancer Res. 49*:331 (1989).

Anafi *et al.*, *Journal of Biological Chemistry 267*:4518-4523 (1992) examined the effect of different compounds on *abl* proteins. According to Anafi:

> The ability of some tyrphostins to discriminate between $p210^{bcr-abl}$, $p185^{bcr-abl}$, and $p140^{c-abl}$ suggests that it should, in principle, be possible to achieve even greater selectivity and aim for tyrphostins with high affinities toward the oncogenic forms of *abl* kinases.

SUMMARY

The present invention concerns methods and compounds for inhibiting cell proliferative disorders characterized by abnormal *abl* activity. The preferred compounds described herein inhibit cell proliferative disorders by targeting abnormal *abl* activity. The preferred target is abnormal *abl* autokinase activity. However, other mechanisms involving *abl* activity may be responsible for the observed cell proliferation inhibition described in the examples below. For example, the compounds may interact with abnormal *abl* substrates, such as Grab-2 (Pendergast, *et al.*, *supra*), and, thus, inhibit the effect of abnormal *abl* activity.

As would be appreciated by one skilled in the art, the compounds described herein have other uses such as being used as lead structures for obtaining additional compounds having equivalent or better activity, screening for additional compounds having equivalent or better activity, and in helping to diagnose if a cell proliferative disorder is caused by abnormal *abl* activity.

Different groups of compounds whose members can inhibit growth of cells characterized by abnormal *abl* activity are described herein. By "characterized by" is meant that abnormal *abl* activity is present in a cell. In addition, inhibition of the abnormal *abl* activity, or the effect of the abnormal *abl* activity, will to some extent inhibit growth of the cell having the abnormal *abl* activity. Also described are examples of compounds belonging to these different groups; examples demonstrating the ability of exemplary compounds to inhibit cell proliferation of cells characterized by abnormal *abl* activity, induce differentiation of cells characterized by abnormal *abl* activity, and/or inhibit *bcr-abl* autokinase ability. Additionally, guidelines for obtaining other members of the different groups able to either inhibit cell growth, induce differentiation, or inhibit *bcr-abl* autokinase activity are described. Thus, the present disclosure provides sufficient information for one skilled in the art to obtain other members of the different groups useful in the present invention.

"Cell proliferative disorders" refer to disorders wherein unwanted cell proliferation of one or more subset(s) of cells in a multicellular organism occurs, resulting in harm (e.g., discomfort or decreased life expectancy) to the multicellular organism. Cell proliferative disorders can occur in different types of animals and in humans. Cell proliferative disorders include cancers, such as chronic myelogenous leukemia and acute lymphoblastic leukemia.

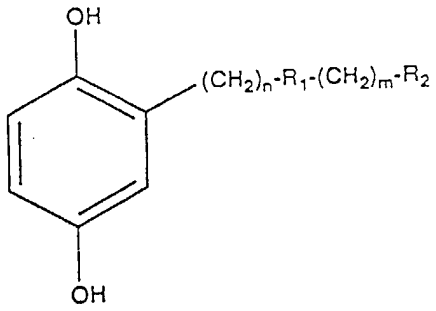
The preferred use of the described compounds is as a therapeutic agent in the treatment of a cell proliferative disorder. Therapeutic agents should be administered in a dosage sufficient to have a therapeutic effect. A therapeutic effect is achieved by eliminating or inhibiting the growth, to some extent, of cells causing or contributing to a cell proliferative disorder. A therapeutic effect relieves to some extent one or more of

the symptoms of a cell proliferative disorder. In reference to the treatment of a cancer, a therapeutic effect refers to one or more of the following: 1) reduction in tumor size; 2) inhibition (i.e., slowing to some extent, preferably stopping) of tumor metastasis; 3) inhibition, to some extent, of tumor cell growth; and/or 4) relieving to some extent one or more of the symptoms associated with the disorder.

When used as a therapeutic agent, the compounds described herein are preferably administered with a pharmacologically acceptable carrier. A pharmacologically acceptable carrier is a formulation to which the compound can be added to dissolve it or otherwise facilitate its administration. Examples of pharmacologically acceptable carriers include water, saline, physiologically buffered saline, and cyclodextrins. Hydrophobic compounds are preferably administered using a carrier. A factor in choosing an appropriate pharmacologically acceptable carrier is choosing a carrier in which the compound remains active or the combination of the carrier and the compound produces an active compound.

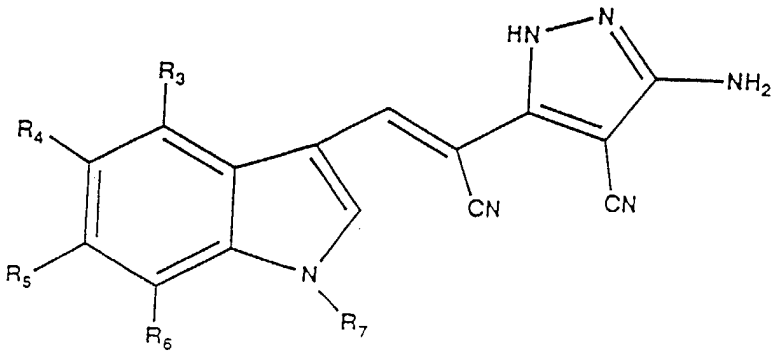
Thus, in a first aspect an agent for treating a patient having a cell proliferative disorder characterized by abnormal *abl* activity is described. The agent which can inhibit growth of a cell having abnormal *abl* activity is selected from the group consisting of:

a) a compound having the chemical formula:



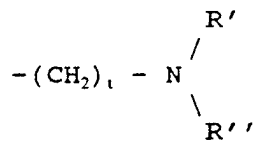
where R_1 is selected from the group consisting of NH, O, and S, R_2 is substituted phenyl having 1 to 3 substituents selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino, n is 0 or an integer between 1 and 6, and m is 0 or an integer between 1 and 6, provided that if n is 1 and m is 0 said substituted phenyl is not 2-$CO(NH_2)$-phenyl or 4-$(COOCH_3)$-phenyl;

b) a compound having the chemical formula:



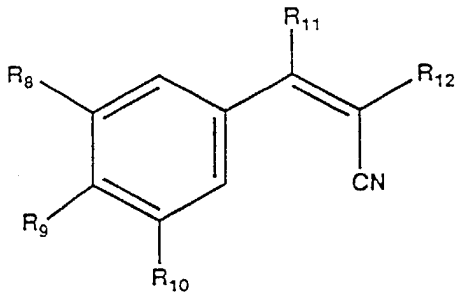
where R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy,

alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2, and NH_2; and R_7 is either H or has the chemical formula:



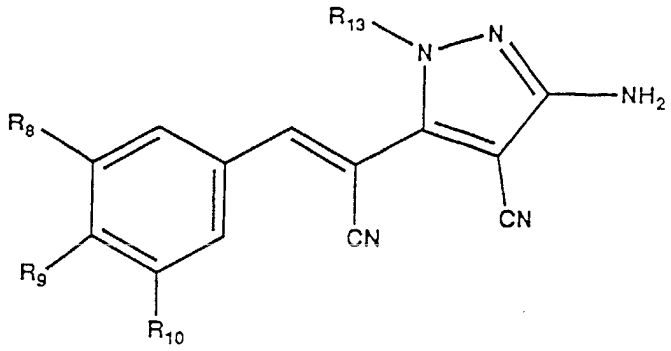
where t is an integer between 1 and 12, and R′ and R′′ is each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, alkoxy and aryl;

c) a compound having the chemical formula:



where R_8, R_9, and R_{10}, is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is an alkylaryl; and R_{12} is selected from the group consisting of further substituted aryl, aryl, CN, amide, and thioamide,

d) a compound having the chemical formula:



where R_8, R_9, and R_{10} is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen hydrogen, NO_2 and NH_2; R_{11} is H; and R_{13} is a substituted phenyl having 1 to 3 substituents selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino, or phenyl;

e) AIV-41; and

f) AIV-42.

"Abnormal *abl* activity" refers to a change in one or more *abl* activities compared to that of a normal *abl* protein, and includes the following: 1) an increase in kinase activity; 2) a different substrate specificity; 3) a different cellular location; and/or 4) a different duration of signal. (Anafi, M., *et al.*, *J. Biol. Chem.* *267*:4518-4523, 1992). A normal *abl* protein is that occurring in the general population which is not associated with a cell proliferative disorder. An abnormal *abl* protein has one of the following abnormalities (compared to a normal *abl* protein): 1) fusion with another protein, such as, for example, *bcr*; 2) truncation; 3) other mutations such as, for example, amino acid substitutions and internal deletions.

Methods of inhibiting abnormal *abl* activity such as p210 *bcr-abl* or p185 *bcr-abl* activity includes targeting the abnormal protein autokinase activity; and inhibiting phosphorylation of substrates by the abnormal protein, particularly those substrates not phosphorylated by normal *abl*. Effective compounds targeted to inhibit the tyrosine phosphorylation of a tyrosine kinase, such as p210 *bcr-abl*, may also act by causing the production of an agent which inhibits cell proliferation (Anafi *et al.*, *FEBS 330*:260, 1993). In preferred embodiments, the compound inhibits abnormal *abl* activity due to a *bcr-abl* fusion, such as p210 *bcr-abl* or p185 *bcr-abl*.

The compounds targeted to cell proliferative disorders resulting from *bcr-abl* fusions preferably inhibit the kinase ability of an isolated *bcr-abl* fusion as measured by the methods described herein. *In vitro* inhibition refers to an IC_{50} (dose required for 50% inhibition) of 50 μM or less, more preferably 5 μM or less, even more preferably 1 μM or less. More preferably, the compound inhibits the kinase ability of the *bcr-abl* fusion in whole cells with an IC_{50} of 50 μM or less, more preferably 5 μM or less, even more preferably 1 μM or less. Compounds with effective *in vitro* are good candidates for therapeutic compounds. The activity of those compounds effective *in vitro* can be confirmed using animal models. For example, Gishizky, M, *et al.*, *Proc. Natl. Acad. Sci. USA 90*:3755-3759 (1993) describes such a model for transplantation of *bcr-abl* induced chronic myelogenous leukemia-like syndrome in mice.

Compounds which preferentially inhibit the tyrosine kinase activity of an abnormal *bcr-abl* fusion are preferred compounds for use as a therapeutic in the treatment of cell proliferative disorders characterized by a *bcr-abl* fusion, and use for diagnostic purposes. "Preferentially inhibition" refers to at least a two fold, preferably 5 fold, more preferably 10 fold, greater inhibition on *bcr-abl* activity compared to the total

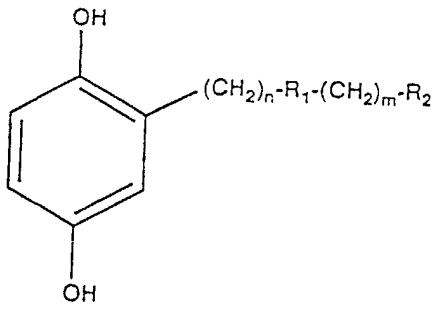
tyrosine kinase activity or epidermal growth factor receptor (EGF-R) activity. Such compounds are preferred because their use in a patient can reduce adverse side reactions resulting from using compounds having a wide range of activities on cellular processes. In addition, such compounds may be used to determine if a disorder is to some extent driven by abnormal *bcr-abl* activity, by assessing the effect of the compound on total tyrosine kinase activity and on EGF-R activity.

In another aspect, a compound selected from the group of compounds consisting of AI-10, AI-11, AI-12, AI-14, AI-15, AII-20, AII-21, AII-22, AIII-35, AIII-37, AIV-41, and AIV-42 is described. These compounds have been found to inhibit growth of cells having abnormal *abl* activity. Of these compounds, compounds AI-10, AI-11, AI-12, AI-14, AI-15, and AII-20 are preferred compounds. These preferred compounds have a strong inhibitory effect on p210 *bcr-abl* kinase activity.

In another aspect, a composition containing a therapeutically effective amount of a compound mentioned above, and a pharmacologically acceptable carrier is described.

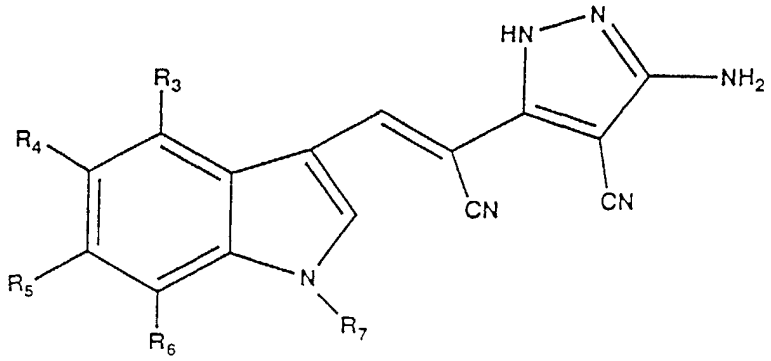
In another aspect a method of treating a patient having a cell proliferative disorder characterized by abnormal *abl* activity is described. The method involves administering to the patient a therapeutically effective amount of a compound selected from the group consisting of:

a) a compound having the chemical formula:

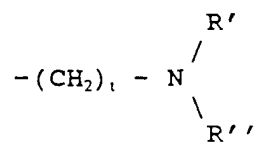


where R_1 is selected from the group consisting of NH, O, and S, R_2 is an aryl, n is 0 or an integer between 1 and 6, and m is 0 or an integer between 1 and 6;

b) a compound having the chemical formula:

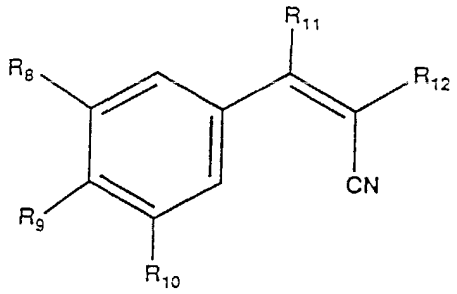


where R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2, and NH_2; and R_7 is either H or has the chemical formula:



where t is an integer between 1 and 12, and R′ and R′′ is each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, alkoxy and aryl;

c) a compound having the chemical formula:



where R_8, R_9, and R_{10}, is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is selected from the group consisting of an H, alkyl, and alkylaryl; and R_{12} is selected from the group consisting of aryl, further substituted aryl, CN, amide, and thioamide,

d) AIV-40;

e) AIV-41; and

f) AIV-42.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the chemical structures of Groups I, II and III respectively.

Figure 2 illustrates the chemical structure of Group IIIa compounds.

Figures 3A-D illustrate the chemical structures of exemplary compounds belonging to Groups I-IV.

DETAILED DESCRIPTION OF THE INVENTION

The present invention features compounds and methods for inhibiting cell proliferative disorders characterized by abnormal *abl* activity. Data is presented below illustrating the ability of exemplary compounds, belonging to different compound Groups, to inhibit the growth of cells characterized by abnormal *abl* activity (i.e., chronic myelogenous leukemia K562 cells). The preferred compounds are those compounds which can inhibit the kinase activity of abnormal *abl* proteins. Using the present application as a guide, one skilled in the art can obtain other compounds having equivalent or better activity.

I. Chemical Definitions

The following is a list of some of the definitions used in the present disclosure.

An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, halogen, alkoxy, =O, =S, NO_2, $N(CH_3)_2$, amino, or SH.

An "alkenyl" group refers to an unsaturated hydrocarbon group containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, halogen, $N(CH_3)_2$, amino, or SH.

An "alkynyl" group refers to an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and

cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, $N(CH_3)_2$, amino or SH.

An "alkoxy" group refers to an "-O-alkyl" group, where "alkyl" is defined as described above.

An "aryl" group refers to an aromatic group which has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, NO_2, amine, thioether, cyano, alkoxy, alkyl, and amino groups. A "further substituted aryl" refers to an aryl in which the preferred substituent(s) include those mentioned above for an aryl and an additional aryl.

An alkylaryl group refers to an alkyl (as described above), covalently joined to an aryl group (as described above).

Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted.

Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted.

An "amide" refers to an -C(O)-NH-R, where R is alkyl, aryl, alkylaryl or hydrogen.

A "thioamide" refers to -C(S)-NH-R, where R is alkyl, aryl, alkylaryl or hydrogen.

An "ester" refers to an -C(O)-OR', where R' is alkyl, aryl, or alkylaryl.

An "amine" refers to a -N(R'')R''', where R'' and R''', is each independently either hydrogen, alkyl, aryl, or alkylaryl, provided that R'' and R''' are not both hydrogen.

A "substituted phenyl" refers to a phenyl having 1 to 3 substituents selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, COOH, and amino.

A thioether refers to -S-R, where R is either alkyl, aryl, or alkylaryl.

II. CELL PROLIFERATIVE DISORDERS

In one aspect the present invention provides methods of inhibiting or decreasing proliferation of cells having enhanced proliferation due to abnormal *abl* activity and compounds useful in these methods. Proliferation of cells, particularly leukemic cells having enhanced proliferation due to abnormal *abl* activity may be inhibited or decreased by exposing the cells to an amount of one of the compounds described herein (see Section III, infra) effective to inhibit or decrease activity of a *bcr-abl* fusion protein. Thus, the present invention has application in different self-proliferative disorders characterized by abnormal *abl* activity, such as hematopoietic cell proliferative disorders including forms of leukemia. Blood cells such as T and B lymphocytes, granulocytes, macrophages, mast cells, megakaryocytes, erythrocytes and eosinophils originate from a self-renewing population of multi-potential hemopoietic stem cells, located mainly in the bone marrow, which generate progenitor cells committed irreversibly to one or another of the various hemopoietic lineages. Progenitor cells, in turn, may each generate clones of lineage-restricted cells that mature into specialized cells. A variety of cytoplasmic tyrosine kinases are expressed in, and may have important functions in, hematopoietic cells including src, lyn, fyn, blk, lck, csk

and hck. (Eisenian, E. and J.B. Bolen, *Cancer Cells 2(10)*:303-310, 1990). T-cell activation, for example, is associated with activation of lck. The signaling activity of lyn may be stimulated by binding of allergens to IgE on the surface of basophils. (Eisenian, supra).

Abnormalities in tyrosine kinase regulated signal transduction pathways can result in hematopoietic cell proliferative disorders. For example, mutations in the cytoplasmic tyrosine kinase *atk* are responsible for the agammaglobulinemia, (Ventrie, D., *et al, Nature 361*:226, 1993). This defect appears to prevent the normal differentiation of pre-B cells to mature circulating B cells and results in a complete lack of serum immunoglobulins of all isotypes.

As illustrated by the ability of the exemplary compounds to inhibit growth of K562 cell line, originally established from a patient with chronic myelogenous leukemia in the terminal blast crisis stage, the present invention is directed to methods and compounds particularly useful for treating leukemia characterized by abnormal *abl* activity. "Leukemia" refers to a progressive proliferation of abnormal leukocytes found in hemopoietic tissues, other organs, and usually in the blood in increased numbers (Stedman's Medical Dictionary 25th edition (Hensyl ed. 1990)). Different forms of leukemia are known in the art and include acute promyelocytic, adult T-cell, basophilic, embryonal, eosinophilic, granulocytic, hairy cell, leukopenic, lymphoblastic, lymphocytic, mature cell, megakaryocytic, meningeal, micromyeloblastic, mixed cell, monocytic, myeloblastic, myelomonocytic, neutrophilic, plasma cell, polymorphocytic, Reider cell, splenic, stem cell, and subleukemic.

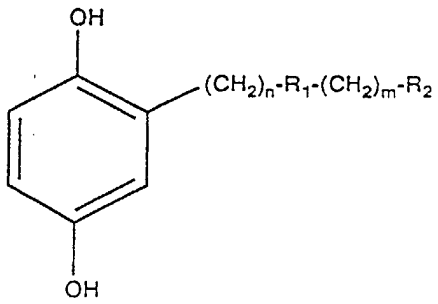
Leukemia's are targeted by the present invention by directly inhibiting cell growth or inducing differentiation. "Differentiation" refers to the maturation process of immature cells. The failure of

cells to properly differentiate can lead to the build up of immature cells resulting in a cell proliferative disorder. The differentiated cells are not immortal. Inducing differentiation results in inhibiting cell growth because the terminally differentiated cells do not proliferate.

III. FEATURED COMPOUNDS

A. Group I compounds

Group I compounds have the general structure:



where R_1 is selected from the group consisting of NH, O, and S, R_2 is aryl, n is an integer between 0 and 6, and m is an integer between 0 and 6;

Examples of Group I compounds are listed in Table I and shown in Fig. 3a.

TABLE 1

Compound	n	R_1	m	R_2
AI-10	1	NH	O	4-COOH-phenyl
AI-11	1	NH	O	2-COOH-phenyl
AI-12	1	S	O	2-COOH-phenyl
AI-13	1	NH	O	2-CO(NH_2)-phenyl
AI-14	1	NH	O	3-CO(NH_2)-phenyl
AI-15	1	NH	O	2-$COOCH_3$-phenyl
AI-16	1	NH	O	4-$COOCH_3$-phenyl

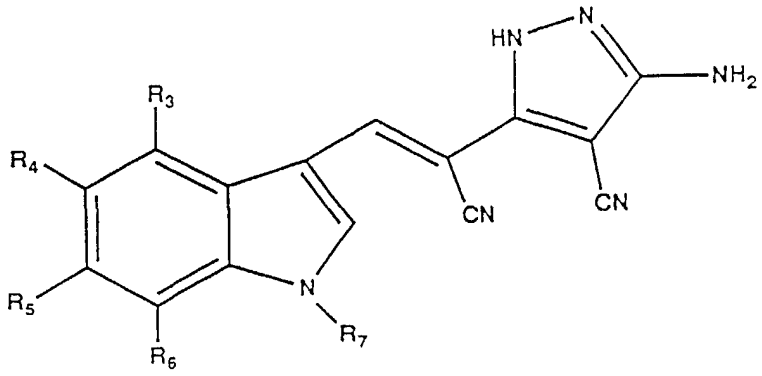
In preferred embodiments n is 1-3, preferably 1; m is 0-3, preferably 0; R_2 is a substituted phenyl having 1 to 3 substituents independently selected from the group consisting of COOH, ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino; preferably R_2 is a substituted phenyl having one substituent selected from the group consisting of $COOCH_3$, COOH, and $CO(NH_2)$; more preferably if n is 1, and m is 0 the substituted phenyl is not 2-$CO(NH_2)$-phenyl or 4-($COOCH_3$)-phenyl. The exemplary Group I compounds shown in Table 1 are all very effective in inhibiting p210 *bcr-abl* kinase activity (see the examples described below).

Examples of novel Group I compounds include AI-10, AI-11, AI-12, AI-14 and AI-15. These novel compounds define a subset of Group I compounds (see, Figure 1, Group I) where R_1 is selected from the group consisting of NH, O, and S, R_2 is a substituted phenyl having 1 to 3 substituents independently selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino, n is an integer between 0 and 6, and m is an integer between 0 and 6, provided that if n is 1, and m is 0, R_2 is not 2-$CO(NH_2)$-phenyl or 4-($COOCH_3$)-phenyl. In preferred embodiments of novel Group I compounds n is 1-3, preferably 1; m is 0-3 preferably 0; R_1 is S or NH, and R_2

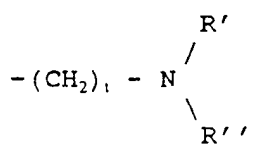
is substituted phenyl containing 1 to 3 substituents independently selected from the group consisting of ester, COOH, and $CO(NH_2)$, preferably R_2 is substituted phenyl having one substituent selected from the group consisting of $COOCH_3$, COOH, and $CO(NH_2)$.

B. Group II Compounds

Group II compounds have the general structure:



where R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2, and NH_2; and R_7 is selected from the group selected from H or:



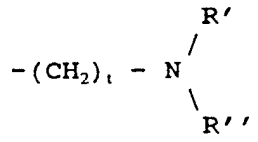
where t is an integer between 1 and 12, and R′ and R′′ is each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, alkoxy and aryl;

Examples of Group II compounds are listed in Table 2 and shown in Figure 3b. The compounds listed in Table 2 have the Group II generic structure where, R_7 is not hydrogen, and R_3, R_4, R_5, and R_6 is hydrogen. An example of a Group II compound where R_7 is H is AII-20.

TABLE 2

Compound	t	R′	R′′
AII-21	3	CH_3	CH_3
AII-22	2	CH_3	CH_3

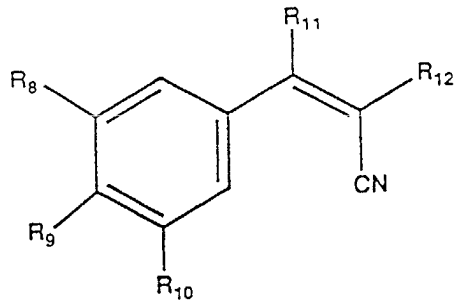
In preferred embodiments R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of hydrogen, alkyl, and OH, preferably H; and when R_7 has the chemical formula:



where t is 1 to 6; and R′ and R′′ is each independently hydrogen, alkyl, or halogen, preferably methyl.

C. Group III Compounds

Group III compounds have the general structure:



where R_8, R_9, and R_{10}, is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is selected from the group consisting of an H, alkyl, and alkylaryl; and R_{12} is selected from the group consisting of aryl, further substituted aryl, CN, amide, and thioamide.

Examples of Group III compounds are listed in Table 3 and shown in Fig. 3c.

TABLE 3

Compound	R_8	R_9	R_{10}	R_{11}	R_{12}
AIII-30	OCH_3	OH	Br	H	CN
AIII-31	OH	OH	H	H	$C(0)NHCH_2$-phenyl
AIII-34	OH	OH	H	H	3-amino 4-cyano pyrazole
AIII-32	OH	OH	H	H	$C(O)NH(CH_2)_3$-phenyl
AIII-33	OH	OH	H	H	$C(O)NH(CH_2)_4$-phenyl
AIII-35	H	OH	H	CH_2-phenyl	CN
AIII-36	OH	OH	OH	H	$C(O)NH_2$
AIII-37	OH	OH	H	H	1-phenyl 3-amino 4-cyano pyrazole

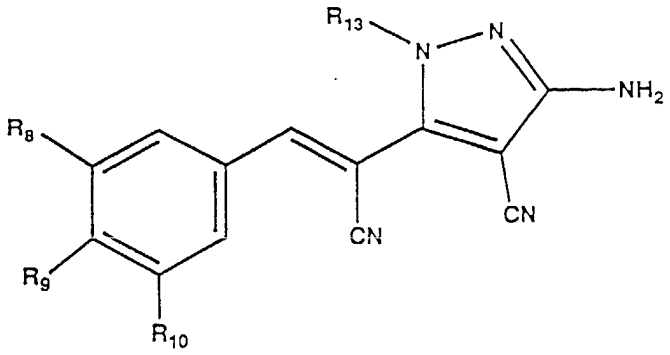
In preferred embodiments R_8 is alkoxy, OH, halogen or H, preferably OCH_3, OH or H; R_9 is alkoxy, OH, halogen or H, preferably OH; R_{10} is alkoxy, OH, halogen or H, preferably OH, H, or Br; R_{11} is H or alkylaryl, preferably H or CH_2-phenyl; and R_{12} is a thioamide or amide having the formula:

$$\begin{matrix} X_3 \\ \| \\ C\text{-}N\text{-}(CH)_r\text{-aryl} \end{matrix}$$

where X_3 is S or O, and r is an integer between 1-12, preferably 1-6, and the aryl is preferably a substituted phenyl.

Two novel subsets of Group III compounds are: 1) those having the generic Figure of Group III where R_8, R_9, and R_{10}, are each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is an alkylaryl; and R_{12} is selected from the group consisting of

aryl, further substituted aryl, CN, amide, and thioamide, and 2) compounds having the chemical formula:



where R_8, R_9, and R_{10} is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is H; and R_{13} is a substituted phenyl independently having 1 to 3 substituents selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino, or phenyl.

III. Additional Compounds

The present disclosure also relates to the identification of other specific compounds belonging to the groups described herein which are useful in the present invention. Identification can be carried out by assaying the ability of a compound to inhibit abnormal *abl* tyrosine kinase activity, and preferably, the ability of the compound to inhibit growth of cells having a cell proliferative disorder characterized by abnormal *abl* tyrosine kinase activity. Such assays can be preformed as described in the art, or as described in the examples below.

Therapeutic compounds should be more potent in inhibiting cell having abnormal *abl* activity than in

exerting a cytotoxic effect. A measure of the effectiveness and cell toxicity of a compound can be obtained by determining the therapeutic index: LD_{50}/IC_{50}. IC_{50}, the dose required to achieve 50% inhibition, can be measured using standard techniques such as those described herein. LD_{50}, the dosage which results in 50% toxicity, can also be measured by standard techniques, such as using an MTT assay as described by Mossman *J. Immunol. Methods* *65*:55-63 (1983), by measuring the amount of LDH released (Korzeniewski and Callewaert, *J. Immunol. Methods* *64*:313 (1983); Decker and Lohmann-Matthes, *J. Immunol. Methods* *115*:61 (1988), or by measuring the lethal dose in animal models. Compounds with a large therapeutic index are preferred. The therapeutic index should be greater than 2, preferably at least 10, more preferably at least 50.

Animal model systems known in the art and deemed predictive of human *in vivo* activity can be used to further confirm the therapeutically effective compounds belonging to the groups described herein. For example, Gishizky M., *supra*, describes transplantation of *bcr-abl* induced myelogenous leukemia-like syndrome in mice. The mice described by Gishizky *et al.*, can be used as an animal model for *bcr-abl* induced myelogenous leukemia. Another example of an animal model is described by Heisterkamp, N., *et al.*, *Nature* *344*:251-251, 1990. Heisterkamp *et al*, describes a transgenic model in which mice expressing a *bcr-abl* mutant protein develop lymphoid malignancies.

In addition to measuring tumor growth in the animal models, plasma half-life and bio-distribution of the drug and metabolites in plasma, tumors, and major organs can be determined to facilitate the selection of drugs most appropriate for the inhibition of a disorder. Such measurements can be carried out, for example, using HPLC analysis on extracts of tissues or blood of treated animals. Compounds that show potent inhibitory activity in the screening assays but have poor pharmacokinetic

characteristics can be optimized by altering the chemical structure to produce additional compounds, preferably within the described groups. The additional compounds can be test. In this regard, compounds displaying good pharmacokinetic characteristics can be used as models.

Toxicity studies can also be carried out by measuring the blood cell composition. For example, toxicity studies can be carried out as follows: 1) the compound is administered to mice (an untreated control mouse should also be used); 2) blood samples are periodically obtained via the tail vein from one mouse in each treatment group; and 3) the samples are analyzed for red and white blood cell counts, blood cell composition, and the percent of lymphocytes versus polymorphonuclear cells. A comparison of results for each dosing regime with the controls indicates if toxicity is present.

At the termination of each study, further studies can be carried out by sacrificing the animals (preferably, in accordance with the American Veterinary Medical Association guidelines Report of the American Veterinary Medical Assoc. Panel on Euthanasia. *Journal of American Veterinary Medical Assoc.*, *202*:229-249, 1993). Representative animals from each treatment group can then be examined by gross necropsy for immediate evidence of metastasis, unusual illness, or toxicity. Gross abnormalities in tissue are noted, and tissues are examined histologically. Compounds causing a reduction in body weight or blood components are less preferred, as are compounds having an adverse effect on major organs. In general, the greater the adverse effect the less preferred the compound.

IV. Administration Of Featured Compounds

The compounds of this invention can be administered to a patient preferably in a pharmaceutical composition comprising the active compound and a carrier or excipient. The compounds also can be prepared as

pharmaceutically acceptable salts (i.e., non-toxic salts which do not prevent the compound from exerting its effect).

Pharmaceutically acceptable salts can be acid addition salts such as those containing hydrochloride, sulfate, phosphate, sulfamate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, *p*-toluenesulfonate, cyclohexylsulfamate and quinate. (*See*, e.g., *supra*. PCT/US92/03736). Such salts can be derived using acids such as hydrochloric acid, sulfuric acid, phosphoric acid, sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, *p*-toluenesulfonic acid, cyclohexylsulfamic acid, and quinic acid.

Pharmaceutically acceptable salts can be prepared by standard techniques. For example, the free base form of the compound is first dissolved in a suitable solvent such as an aqueous or aqueous-alcohol solution, containing the appropriate acid. The salt is then isolated by evaporating the solution. In another example, the salt is prepared by reacting the free base and acid in an organic solvent.

Carriers or excipient can be used to facilitate administration of the compound, for example, to increase the solubility of the compound. Examples of carriers and excipients include calcium carbonate, calcium phosphate, various sugars or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols and physiologically compatible solvents. The compounds or pharmaceutical composition can be administered by different routes including intravenously, intraperitoneally, subcutaneously, and intramuscularly; orally, topically, or transmucosally.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ring-

er's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, many small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the

effective amounts is within the capability of those skilled in the art in light of the detailed disclosure provided herein.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained, for example by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

Another example of a pharmaceutically acceptable carrier is PBTE. PBTE consists of a solution of 3% w/v benzyl alcohol, 8% w/v polysorbate 80, and 65% w/v polyethylene glycol (MW = 300 daltons) in absolute ethanol. PBTE:D5W consists of PBTE diluted 1:1 in a solution of 5% dextrose in water.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture and animal models. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

The use of hydrophobic compounds can be facilitated by different techniques such as combining the compound with a carrier to increase the solubility of the

compound and using frequent small daily doses rather than a few large daily doses. For example, the composition can be administered at short time intervals, such as by the methods described above or using a pump to control the time interval or achieve continuous administration. Suitable pumps are commercially available (e.g., the ALZET® pump sold by Alza corporation, and the BARD ambulatory PCA pump sold by Bard MedSystems).

The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used, and the size and physiological condition of the patient. For the treatment of cancers the expected daily dose is between 1 to 2000 mg/day, preferably 1 to 250 mg/day, and most preferably 10 to 150 mg/day. Drugs can be delivered less frequently provided plasma levels of the active moiety are sufficient to maintain therapeutic effectiveness.

A factor which can influence the drug dose is body weight. Drugs should be administered at doses ranging from 0.02 to 25 mg/kg/day, preferably 0.02 to 15 mg/kg/day, most preferably 0.2 to 15 mg/kg/day. Alternatively, drugs can be administered at 0.5 to 1200 mg/m^2/day, preferably 0.5 to 150 mg/m^2/day, most preferably 5 to 100 mg/m^2/day. The average plasma level should be 50 to 5000 $\mu g/ml$, preferably 50 to 1000 $\mu g/ml$, and most preferably 100 to 500 $\mu g/ml$. Plasma levels may be reduced if pharmacological effective concentrations of the drug are achieved at the site of interest.

V. Examples

Examples are provided below to illustrate different aspects and embodiments of the present invention. These examples are not intended in any way to limit the disclosed invention. Rather, they illustrate methodology by which drugs having the disclosed formulae can be readily identified by routine procedure to ensure that they have the desired activity, and the synthesis of

different compounds described herein. Compounds within the different formulas claimed herein can be screened to determine those with the most appropriate activity prior to administration to an animal or human. Other compounds can also be screened to determine suitability for use in methods of this invention.

Example 1: Compounds which induce differentiation:

This example describes compounds which induce differentiation in cells having abnormal *abl* activity and techniques which can be used to obtain additional compounds able to induce differentiation and/or inhibit cell proliferation belonging to the different groups of compounds described herein.

Materials & Methods

Cell and Culture conditions:

The K562 cell line was originally established from a pleural effusion of a chronic myelogenous leukemia (CML) patient in the terminal blast crisis stage. Cells were cultured in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 10 g/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere, with 5% CO_2 in air.

Assay for Cell Growth and erythroid Differentiation:

Cells (about 4 X 10^5 cells/ml) were incubated in various concentrations of compound in a final volume of 2 ml dimethylsulfoxide (DMSO). Untreated control cells were treated with 2 ml DMSO. The number of live cells, and cell mortality levels were measured by staining the cells with crystal violet. The erythroid differentiation of the cells was evaluated by benzidine staining.

Analysis of Proteins containing Phosphotyrosine in Treated and Untreated Cells:

Exponentially growing K562 cells (approximately 7 X 10^5 cells/ml), were treated with non-toxic levels of

compounds (up to 100 μM). The cells were then washed twice with HANKS buffered solution, and the pellet was resuspended with protein lysis buffer containing 10 mM Tris pH 8, 50 mM NaCl, 5 mM EDTA, 30 mM Na-pyrophosphate, 50 mM NaF, 1% Triton X-100, 2 mM phenylmethylsolfonyl fluoride, 100 KIU/ml aprotinin, 5μg/ml leupeptin and 100 μM sodium vanadate. Cell lysates were prepared on ice for 1 hour with short vortexing every 10 minutes. Cell lysates were cleared by centrifugation at 12,000 g for 30 minutes. The protein concentrations of the supernatants were measured using a Bio-Rad™ protein assay, and the cell lysate was boiled for 6 minutes in SDS gel sample buffer. Extracts (60 μg) were loaded onto different lanes of 7.5% SDS polyacrylamide gel, electrophoresed and blotted to nitrocellulose paper (0.2 μ, Schleicher and Schuell Inc.). Protein blots were blocked overnight with TBST (50 mM Tris base pH 7.5, 150 mM sodium chloride and 0.05% Tween-20), 5% bovine serum albumin and 1% chicken egg albumin, then incubated for 2 hours at room temperature with phosphotyrosine antibodies (Zymed Inc.) in blocking solution. The filters were washed and incubated with protein A-peroxidase for 40 minutes at room temperature, washed again and subjected to ECL reaction (Amersham Inc.) and autoradiography.

Stripping of the filters for further antibody reaction was done at 50°C in 10 mM Tris, pH 7.5, 2% SDS and 100 mM 2-β-mercaptoethanol. The filters were reincubated for 2 hours at room temperature with monoclonal anti-*abl* antibodies. Equivalent anti-*abl* antibodies are known in the art or can be obtained using standard techniques (See, e.g., Guo, J.Q., *et al. Cancer Res.* 51(1):3048-3051 (1991)). Finally, the filters were washed and incubated with peroxidase conjugated goat anti-mouse antibodies for 40 minutes at room temperature, washed again and subjected to ECL reaction and autoradiography.

In Vitro Tyrosine Kinase Assays:

The assays were performed using the copolymer $Glu_6Ala_3Tyr_4$ (Sigma Inc.) as previously described by Anafi *et al.*, *J. Biol. Chem.* 267:4518 (1992) and Yaish *et al.*, *Science* 242:933 (1988).

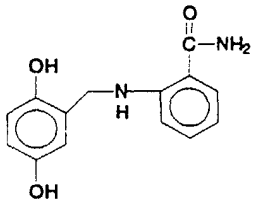
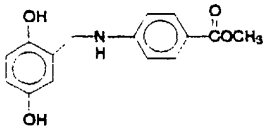
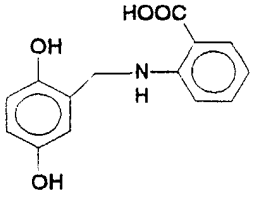
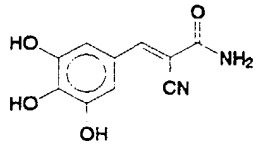
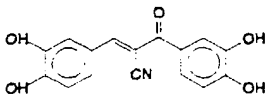
B. Results

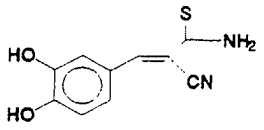
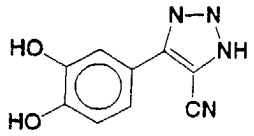
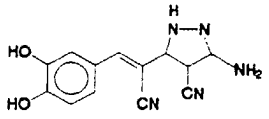
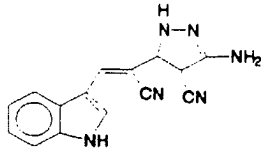
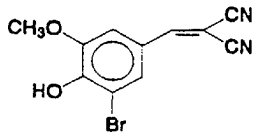
Compound Screening for the Ability to Induce Differentiation:

Various compounds were checked for their ability to induce erythroid differentiation of K562 cells (Table 4). Four compounds were able to induce over 50% of the cells in the culture to differentiate. The four compounds were further analyzed (Table 5). The IC_{50} values of compound for p210 *bcr-abl* and EGF-R were determined as described by Anafi et al., *supra*, and Yaish *et al.*, *supra*, respectively. Using the methods described herein, one skilled in the art can select for those compounds which can induce differentiation and which are useful in the treatment of a cell proliferation disorder.

TABLE 4

Screening of *in vitro* active tyrphostins for their ability to differentiate K562 cells

TYRPHOSTINS	Structure	IC_{50}, (μM) p210$^{ber-abl}$	IC_{50}, (μM) EGF receptor	Inducer for K562 differentiation
AI-13		0.77±0.23	0.28	-
AI-16		1±0.14	0.25	-
AI-11		1.9±0.2	0.58	-
AIII-36		2.7±0.3	2.8	-
		4±0.5	0.37	-

TYRPHOSTINS	Structure	IC_{50}, (μM) $p210^{bcr-abl}$	IC_{50}, (μM) EGF receptor	Inducer for K562 differentiation
		5.8±0.9	2.4	-
AIV-40		1.3±0.2	94	+
AIII-34		1.8±0.4	1.1	+
AII-20		10.2±3.5	18.5	+
		>>20	143	+

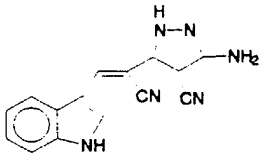
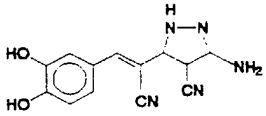
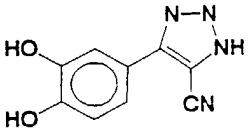
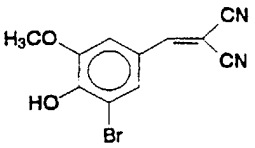
"-" refers to less than 50% induction of K562 cells.

"+" refers to over a 50% induction of K562 cells.

35/1

TABLE 5

Erythroid induction of K562 cells by compounds.

TYRPHOSTINS Name	Structure	Induction of erythroid differentiation (ED_{50}, μM)	Minimal toxic concentration (MTC, μM)	ED_{50}/MTC	Tyrosine kinase inhibition activity in cells
AII-20		2	>67	>34	+
AIII-34		20	200	10	+
AIV-40		50	>200	>4	-
AIII-30		25	100	4	-

The effective dose of 50% of maximal percentage of differentiation (ED_{50}) was calculated from dose response curve of K562, treated with different compound concentrations. The minimal toxic concentration (MTC) is the first concentration in which the cells were arrested or killed prior to their differentiation.

As illustrated in Table 5, AII-20, AIII-30, AIII-34, and AIV-40 were more effective in inducing erythroid differentiation than killing cells (i.e., ED_{50} < minimal toxic concentration). AIII-30 and AIV-40 did not significantly inhibit tyrosine kinase activity in intact K562 cells. The compounds AIII-34 and AII-20 were found

to induce erythroid differentiation as well as to block tyrosine phosphorylation in the intact cells.

The affects of AII-20 and AIII-34 on the onset of erythroid differentiation and cell growth were further investigated. Compounds AII-20 and AIII-34 were added to cells and the onset of erythroid differentiation was followed for 8 days in parallel with cell growth measurements. Cell mortality throughout the experiment for treated and untreated cultures was about 5%. From day 3, cell differentiation was accompanied by growth arrest, and its degree correlated to the level of cell differentiation. After one day of treatment all the AII-20 concentrations tested, and up to 100 μM AIII-34, had no effect on cell proliferation and just a small effect on differentiation. After five days of treatment, the differentiation level reached 60% in the treated cells (differentiation was less than 1% in untreated cells). At low compound concentrations a good correlation was found between the degree of differentiation and the induction of growth arrested.

A number of tyrosine phosphorylated proteins were identified in western blots of lysates of K562 cells. Several phosphorylated bands diminished following treatment at concentration of AII-20 and AIII-34, which were effective in inducing cell differentiation along with growth arrest. A 210 kDa band was affected by these compounds and by herbimycin A. Herbimycin is a non-selective phosphotyrosine blocker which can induce K562 differentiation (*Cancer Res.* 49:331 (1989)). Herbimycin A treatment resulted in a concomitant decrease of the 210 kDa protein detected with monoclonal anti-*abl* antibodies (8E9), while no similar decrease was evident following treatment with AII-20 or AIII-34. Stripping experiments confirmed the identity of the phosphorylated p210 band as p210 *bcr-abl*. Differentiation of K562 is therefore associated with reduced phosphorylation of p210 *bcr-abl* consistent with diminished kinase activity.

Inhibition of tyrosine phosphorylation was apparent within 10-30 minutes of AII-20 (67 μM) and AIII-34 (100 μM) treatment. The effect of herbimycin A (0.5 μg/ml) on tyrosine phosphorylation is slower and appears only after 2 hours after it is administered.

AIV-40 and AIII-30 at concentrations which induce erythroid differentiation of K562 cells, do not induce inhibition of phosphorylation of cellular proteins in K562 cells. Two compounds AII-20 and AIII-34 were found to induce erythroid differentiation of K562, and to inhibit the tyrosine phosphorylation of p210 *bcr-abl*, as well as the phosphorylation of other cellular proteins. Inhibition of tyrosine phosphorylation is apparent within one hour, where as differentiation towards the erythroid lineage begins to take effect after two days.

AII-20 and AIII-34 are potent blockers of p210 *bcr-abl* phosphotyrosine kinase in intact K562 cells. These two compounds are believed to be the first compounds for which a direct correlation has been shown for p210 *bcr-abl* phosphotyrosine kinase inhibitory activity and ability to induce erythroid differentiation. AII-20 is a potent inhibitor for at least two tyrosine kinases, p210 *bcr-abl* and EGF receptor.

Example 2: Inhibition of Kinase Activity and Cell Proliferation

This example describes compounds which cell proliferation of cell having abnormal *abl* activity and techniques which can be used to obtain additional compounds able to induce differentiation and/or inhibit cell proliferation belonging to the different groups of compounds described herein.

A. Methods & Materials

Cell culture and cell growth assay:

K562 cells (ATCC 562, Rockville, MD) were cultured in medium (RPMI medium containing 10% fetal calf serum, 2 mM glutamine) containing 100 units/ml penicillin

and 100 μg/ml streptomycin). Cells were transferred to 96 well plates (2 X 10^3 cells/well) and incubated with increasing concentrations of compounds to a final volume of 200 μl. Control cells were incubated with medium containing identical concentrations of the compound solvent (DMSO). Growth of K562 cells were measured after 6 days by measuring the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product (Mossman T., *J. Immunol. Methods* 65:55 (1983)).

Reversibility of Compound effect: Cells were exposed to 15 μM and 25 μM concentrations of the indicated compounds for time periods of 1 hour, 4 hours, and 24 hours. After each time period, cells were washed three times with medium and resuspended in fresh medium. Cells were counted with a hemocytometer and plated in 6 well plates (2 ml, 6,250 cells/ml). Cells for continuous exposure to drug were plated identically in medium with the appropriate drug concentration. Cells were counted by hemacytometer on day 6 and were checked for viability using trypan blue.

Macromolecular synthesis: K562 cells were plated at a density of 5,000 cells per well in 96 well plate in 100 μl of medium. Cells were exposed to compound for the indicated periods and pulsed with [^{3}H]-thymidine, L-[^{3}H]-leucine, and [^{3}H]-uridine at 5 μCi/ml for the last 2 hours of drug exposure or 10 μCi/ml for the last 30 minutes of the drug exposure. Cells were harvested and incorporation of label was assessed as described in Kaur *et al.*, *J. National Cancer Institute* 84:1736-40 (1992).

ATP Levels: Ten million cells were collected by centrifugation and washed once with phosphate buffered saline (PBS). To the cell pellet was added 500 μl of 60% methanol. The contents were mixed, heated at 95°C for 1.5 minutes, clarified by centrifugation and analyzed by ion-exchange HPLC on Partisal SAX column using gradient

elution with ammonium phosphate buffers (Ford *et al.*, *Cancer Res.* 51:3733-40, (1991).

Cell extraction and p210 bcr-abl immunoprecipitation: Exponentially growing K562 cells (1 x 10^7 cells) were washed twice in phosphate buffered saline, and then the cell pellet was lysed in 1.0 ml of ice-cold kinase-lysis buffer (10 mM Na_2HPO_4-NaH_2PO_4 [pH 7.0] 1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS] 150 mM NaCl containing 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml of Aprotinin, and 10 μg/ml of pepstatin), briefly vortexed and centrifuged at 35,000 rpm for 90 minutes. To the clear cell extract leupeptin was added to a final concentration of 50 μg/ml. Each 1 ml of clarified extract was incubated with 5 μl of anti-*bcr-abl* sera (Ab-2, Oncogene Science) or with antiserum which had been incubated with immunizing peptide (10X) at room temperature for 2 hours prior to addition to extract. Incubation with antisera was overnight (16 hours) at 4°C with gentle shaking. To harvest the immune complex, 15 μl packed volume of preswollen protein a-sepharose beads (per .1 ml of extract) were added and extracts were incubated for another 2 hours at 4°C with gentle shaking. Beads were pelleted by centrifugation.

In-vitro auto-phosphorylation reaction (kinase activity): The p210 *bcr-abl* protein immunoprecipitates were washed twice with extraction buffer lacking SDS. Precipitates were washed once with 50 mM Tris (pH 7.0) and resuspended in 20 μl of 20 mM PIPES [piperzine-N,N'-bis(2-ethanesulfonic acid] (pH 7.0)-20 mM $MnCl_2$. In some reactions, acid denatured rabbit muscle enolase (5 μg/5 μl) was added as an exogenous substrate for the p210 *bcr-abl* kinase. Five microliters of compound were added at 8X final concentration of each reaction mixture. Reactions were initiated by adding 10μl of [γ-^{32}p]ATP (10 μCi per sample, 3000 Ci/mmole; Amersham Corp.), incubated for 20 minutes at 30°C, stopped by addition of 10 μl of 5X SDS gel loading buffer, heated at 95°C for 5 minutes and

analyzed on 7.5% SDS-polyacrylamide gel electrophoresis and by autoradiography (Laemmeli, UK., *Nature* 227:680-685 (1970)).

^{32}P-orthophosphate labeling, immunoprecipitation and phosphotyrosine immunoblotting: The kinase activity of p210 *bcr-abl* was measured using an anti-phosphotyrosine antibody. 1 X 10^7 cells were exposed to compounds for time periods of 1, 6 and 24 hours. Cells were labeled for 1 hour with 1 mCi of carrier free ^{32}P-orthophosphate in 5 ml phosphate free medium containing 10% dialyzed serum and appropriate concentrations of the drug. Cells were centrifuged at 1,000 rpm for 5 minutes, washed 3 times, and lysed in 600 μl of 10 mM sodium phosphate (pH 7.5), 100 mM NaCl, 5 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100, 0.5% Sodium deoxycholate, 2 mM phenylmethylsulfonly fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Cell lysates were centrifuged at 14,000 rpm for 15 minutes. Supernatant was removed, and proteins determined by the method of Bradford (Bradford, MM., *Anal. Biochem.* 72:420-428); phosphorylated proteins (15μg) were separated by 7.5% SDS-polyacrylamide (Laemilli, *supra*). Six hundred micrograms of labelled cell lysate protein was immunoprecipitated. Immunoprecipitated proteins were separated by 7.5% SDS-polyacrylamide gels and transferred to Immobilin-P in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0), 10% methanol at O.A for 2 hours at 4°C. Phosphotyrosine was detected by western blotting with a mouse monoclonal anti-phosphotyrosine antibody (#05-321, UBI, NY) followed by alkaline phosphatase detection or with analogously prepared unlabelled cell extracts by ^{125}I-protein A.

B. Results

Inhibition of growth and In-Vitro p210 bcr-abl Autokinase Activity:

To correlate K562 growth inhibition with p210 *bcr-abl* kinase inhibition, K562 cells were exposed to

different compounds for six days followed by estimation of cell number using the calorimetric MTT assay. The same compounds were also studied for their capacity to inhibit p210 *bcr-abl* kinase activity in an immune complex autokinase assay from untreated cells. This assay examines the capacity of the p210 *bcr-abl* to phosphorylate itself on tyrosine.

The ability of different compounds to effect p210 *bcr-abl* kinase activity, and K562 growth was measured. All the compounds tested with an IC_{50} for growth of 50 μM or more for K652 growth had only partial or no inhibitory effect on p210 *bcr-abl* autokinase at 50 μM of drug. Compounds having an IC_{50} < 50 μM for growth of K562 cells differed in their ability to inhibit p210 *bcr-abl* kinase activity (Table 6).

TABLE 6

Compound	IC_{50}	p210 *bcr-abl* inhibition
AI-10	50±3	complete
AI-12	46±6	complete
AIV-41	42±5	no effect
AIII-34	32±3	partial
AII-20	35±11	complete
AI-13	35±3	complete
AIII-35	33±7	no effect
AIV-42	32±2	partial
AI-II	30±11	complete
AIII-31	29±4	partial
AI-14	22±6	complete
AII-21	21±3	partial
AIII-36	19±6	complete
AI-15	16±3	complete
AI-16	15±4	complete
AIII-33	14±3	no effect
AII-22	12±3	partial
AIII-32	9.2±2	no effect
AIII-37	8±1	no effect

IC_{50} was measured using the MTT assay. Inhibition was measured using 50 μM compound concentration. Complete refers to 95-100% inhibition. Partial refers to 10-80% inhibition. No effect refers to < 10% inhibition.

Compounds inhibiting growth of K562 cells differed in their ability to inhibit the autokinase activity. Compounds AIII-34, AIV-42, AIII-31, AII-21, AII-22, partially inhibited autokinase activity. Compounds AIV-41, AIII-35, AIII-33, AIII-32, AIII-37, had no effect on autokinase activity. Compounds AI-10, AI-12, AII-20, AI-13, AI-11, AI-14, AIII-36, AI-15, and AI-16 completely inhibit autokinase activity.

When phosphorylation of enolase as an exogenous substrate of p210 *bcr-abl* autokinase was monitored, no compound emerged which was substantially better in inhibiting phosphorylation of the exogenous substrate as compared to autokinase reaction.

Effects of AI-16 and AIII-32

The foregoing experiments suggested inhibition of p210 bcr-abl activity was in some cases related to inhibition of growth. The cellular effects of AI-16 as an example of cell growth and p210 *bcr-abl* kinase-inhibiting compound, and AIII-32 as an example of a compound inhibiting cell growth but not p210 *bcr-abl* kinase were further characterized. To determine if the growth inhibitory action of AI-16 could be related temporarily to inhibition of p210 *bcr-abl* tyrosine kinase activity, the cellular effects of AI-16 and AIII-32 with were determined shortly after drug addition.

Exposure of K562 cells to AI-16 for 24 hours inhibits DNA, protein and RNA synthesis completely at a concentration of 25 μM. AIII-32 inhibits DNA and RNA synthesis by 80% at 25 μM, but protein synthesis is less affected (only ~50% inhibition) even at 50 μM concentration of the drug. Cells whose growth was arrested after 24 hours of exposure of drug were clearly viable as measured by trypan blue exclusion and by capacity to reduce MTT, which depends on intact mitochondrial electron transport (Mossman, *supra*). After 24 hours of exposure to growth inhibitory concentrations of AI-16 and AIII-32, K562 cells maintained comparable levels of ATP with a similar ATP/ADP ratio compared to untreated or vehicle treated cells. Thus, inhibition of cell growth and macromolecular synthesis did not occur with gross alteration of cellular metabolic capacity.

AI-16 appears to manifest growth inhibition in conjunction with an early decrease in DNA synthesis. AI-16 inhibited [^{3}H]thymidine incorporation by 60% or 90% after 2 hours exposure to AI-16 to 20 μm or 40 μM

respectively; [^{3}H]uridine and L-[^{3}H]leucine incorporation were maintained at >80% after 2 hour exposure to the same concentrations of AI-16, and even at 8 hour of exposure to drug, L-[^{3}H]leucine incorporation was largely unaffected while [^{3}H]uridine incorporation was 60% of control.

As AI-16 and AIII-32 are both potential tyrosine kinase antagonists, we examined the effect of the drugs on total protein and p210 *bcr-abl* tyrosine phosphorylation in K562 cells. Neither AI-16 (25 μM) nor AIII-32 (15 μM) after 24 hours of drug exposure inhibited [$^{32}PO_4$] incorporation into total proteins. However, 1 hour after addition of AI-16, (but not AIII-32), there was a decrease in [$^{32}PO_4$] labelling of the p210 *bcr-abl* protein, and also a decrease in the mass of phosphotyrosine detected by anti-phosphotyrosine antibodies using alkaline phosphatase calorimetric or [^{125}I]-protein A detection technique. AI-16 specifically decreased the phosphotyrosine content of immuno-precipitated p210 *bcr-abl*. Both of these changes occur as decrease in DNA synthesis is developing, but before significant decrease in RNA or protein synthesis. Thus, p210 *bcr-abl* tyrosine kinase inhibition may affect a pathway leading to continued DNA synthesis, and by its inhibition AI-16 could then inhibit cell growth. In contrast, AIII-32 does not ever inhibit p210 *bcr-abl* kinase activity even as it inhibits cell growth.

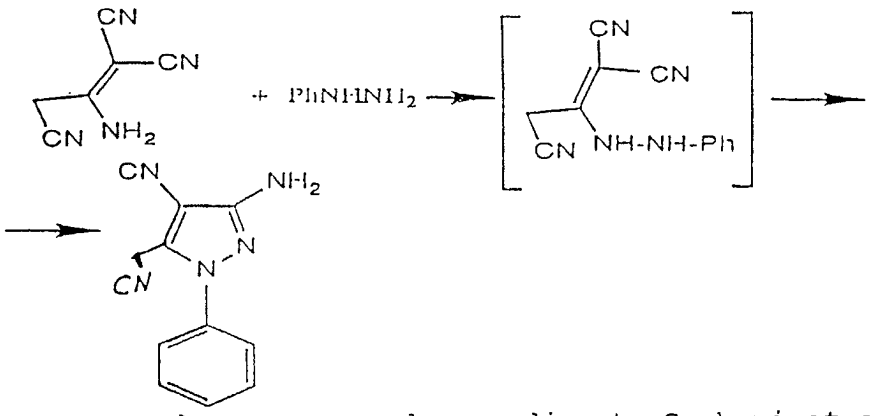
Since a useful therapeutic effect of a compound in CML could be achieved by intermittent exposure to drug, the degree to which K562 cells recover after exposure to AI-16 was assessed. Exposure to AI-16 at 25 μM for 24 hours, or six days of continuous exposure, resulted in analogous growth inhibition. In contrast, exposure for 1 hour or 4 hours demonstrated considerable reversibility of drug effect after washout. AIII-32 was somewhat more reversible at 15 μM and 25 μM after 3 hours of treatment as compared to AI-16. These experiments suggest the use of 10 to 20 μM of AI-16 would be an appropriate concentration for animal models and the use of a treatment

regimen involving a prolonged exposure. Such a exposure can be obtained by standard techniques such as the use of pumps, or continuous administration of the compound.

Chemical Synthesis Examples

Examples of synthesis of exemplary compounds belonging to different groups and classes of compounds are described below. The compounds were generally prepared as 100 μM stock solutions in DMSO and kept at - 70°C. Temperature were measured in degree °C. The stock solutions were diluted to their final concentration in RPMI.

Example 3

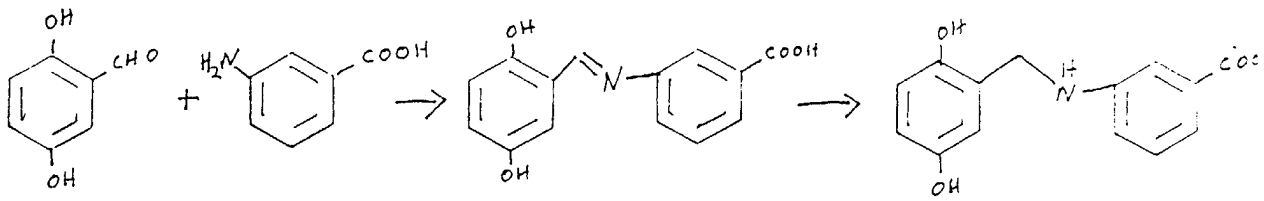


The compound was prepared according to Carboni *et al.*, *J. Am. Chem. Soc.* *80*:2838 (1958). 1.9 g malononitril dimer and 1.65 g phenyl hydrazine in 10 ml methanol were heated 1 hour. Water was added and the solid filtered off and washed with water-methanol to give 0.77 g, 24% yield, pink solid, mp 162°C. NMR acetone d_6 δ7.58 (5H, m), 3.97 (2H, S). mp 166°C.

Example 4

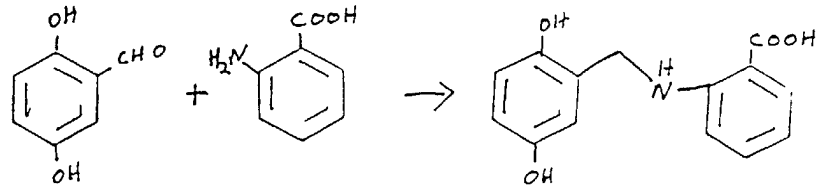
Preparation of Group I Compounds.

A. AI-10



0.41 g, 3 mM, m-amino benzoic acid and 0.41 g, 3 mM, 2.5 Di-OH benzaldehyde in 20 ml CH_3OH were refluxed 16 hours (a red precipitate formed). The reaction was cooled to room temperature (r.t.) and 0.22 g, 3.5 mM, $NaCNBH_4$ was added. After stirring at r.t. 2 hours, it was extracted with EtAc (Extraction with CH_2CI_2 gave traces of material) to give yellow oil which was triturated with $CHCl_3$ and filtered to give 0.2 g, 26% yield yellow solid, mp 145°C. MS 259 (M^+, 9%), 241 ($M-H_2O$, 21), 137 ($M-H-C_6H_4COOH$, 100), 120 (54), m/e.
NMR acetone-d_6 δ 7.40-6.50 (7H, m), 4.34 (2H, s).

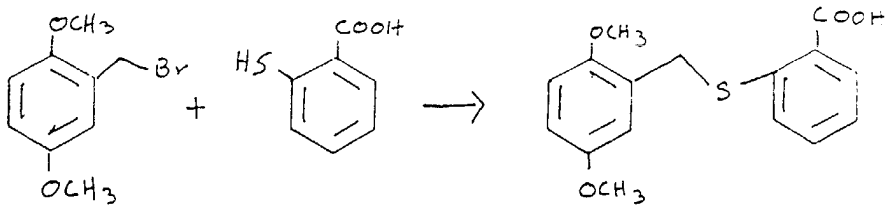
B. AI-11



0.41 g, 3 mM, of each compound in 20 ml CH_3OH were refluxed overnight. The red suspension was cooled to room temperature and 0.3 g $NaCNBH_4$ was added. The color disappeared in 5 minutes. After 1 hour stirring the

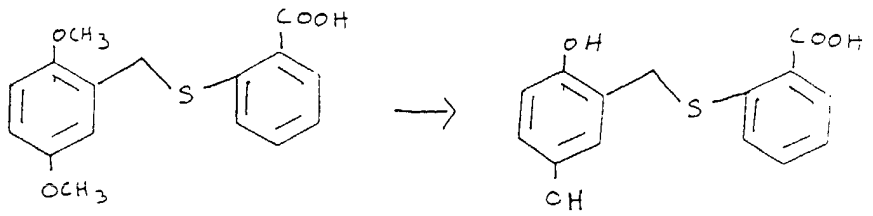
reaction was extracted with EtAc and evaporated to give 0.21 g, 27% yield, lighted-yellow solid, mp 152°C.
MS 259 (M^+, 12%), 215 ($M-CO_2$, 13), 138 ($M-C_6H_4COOH$, 13), 137 (15), 120 (65), 119 (94), 110 (14), m/e.

C. AG 935



To 3.5 g, 16 mM, bromide (see AG 863), in 30 ml ethanol and 10 ml CH_2Cl_2 was added 2.5 g, 16 mM, thiosalicylic acid and 2 ml Et_3N. After 2.5 hours at room temperature the solid was filtered, washed with CH_2Cl_2 and dried to give 2.8 g, 58% yield, white solid, mp 165°C.
NMR acetone d_6, δ 8.01 (1H, m, H_6,), 7.51 (2H, m), 7.20 (1H, m), 7.01 (1H, d, J=3.0 Hz, H_6), 6.94 (1H, d, J=9.0 Hz, H_3), 6.81 (1H, dd, J=9.0, 3.0 Hz, H_4), 4.17 (2H, s, CH_2N), 3.82 (3H, s, OCH_3), 3.71 (3H, s, OCH_3).

D. AI-12

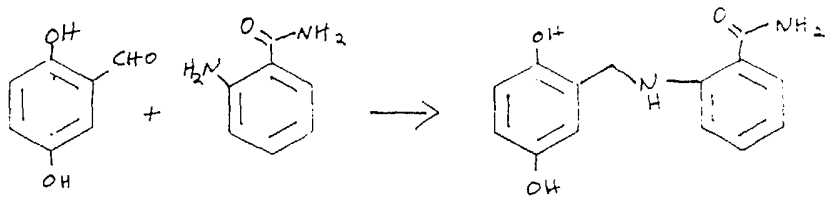


To 1 g, 3.6 mM, AG 935 in 20 ml CH_2Cl_2, under N_2, was added 1.5 ml, 1.5 mM, BBr_3. After 1 hour at room temperature water was added and the reaction extracted with EtAc. Evaporation gave viscous oil which was triturated with CH_2Cl_2 to give 0.28 g, 28% yield, white solid, mp 125°C.

MS 277 (5%), 276 (M^+, 31%), 258 (40), 168 (28), 154 (80%), 138 (37), 137 (80), 136 (100%), 123 (84), 122 (59), 109 (73), 108 (88), m/e.

NMR acetone-d_6 δ 7.98 (1H, m, H_6,), 7.48 (2H, m), 7.20 (1H, m), 6.86 (1H, d, J=2.5 Hz, H_6), 6.72 (1H, d, J=8.6 Hz, H_3), 6.57 (1H, dd, J=8.6, 2.5 Hz, H_4), 4.13 (2H, s, CH_2S).

E. AI-13

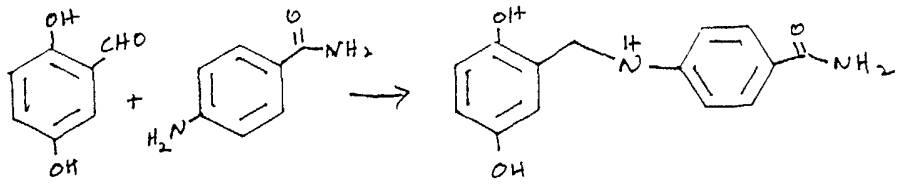


0.5 g, 3.7 mM, of each compound in 30 ml CH_3OH were refluxed 1 hour, cooled and 0.5 g $NaCNBH_4$ was added. Stirring overnight and filtering gave 0.12 g, 12% yield, of white solid, mp 262°C.

MS 258 (M^+, 7%), 256 (30), 254 (100%), 212 (16), 147 (16), 146 (15), 136 (61), 120 (18), 119 (79), m/e.

NMR acetone-d_6 δ 7.78 1H, m, (dd), H_3), 7.28 (1H, m, (dt)), 6.85-6.75 (2H, m), 6.90 (1H, d, J=3.0 Hz, H_6), 6.72 (1H, d, J=8.6 Hz, H_3), 6.65 (1H, dd, J=8.6 Hz, H_4), 6.08 (2H, s).

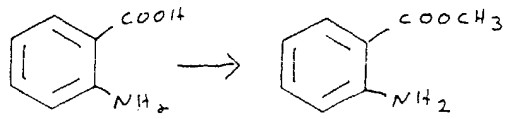
F. AI-14



0.5 g. 3.7 mM, of each compound in 30 ml CH_3OH were refluxed 2 hours to give orange solid. After cooling 0.5 g $NaCNBH_4$ was added. The reaction was stirred 2 hours, evaporated and chromatographed on silica gel to give 0.16 g, 17% yield, white solid.

NMR acetone-d_6 δ 7.73, 6.67 (4H, ABq, J_{AB}=8.8 Hz), 6.75 (1H, d, J=3.0 Hz, H_6), 6.71 (1H, d, J=8.5 Hz, H_3), 6.54 (1H, dd, J=8.5, 3.0 Hz, H_4), 4.34 (2H, s, CH_2N).

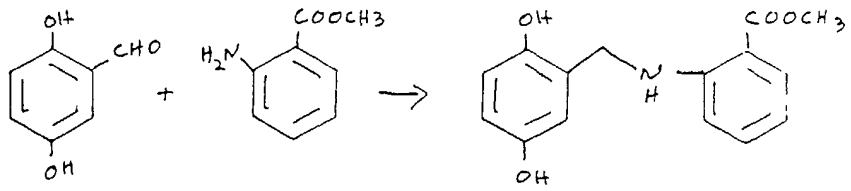
G. AG 949



To 10 g anthranilic acid in 50 ml CH_3OH cooled in ice was added slowly 10 ml $SOCl_2$. Then the reaction was refluxed 2.5 hours, water was added and Na_2CO_3 to neutral pH. Extraction with CH_2Cl_2 gave 4.3 g, 39% yield, light-red oil.

NMR $CDCl_3$ δ 7.82 (1H, m), 7.26 (1H, m), 7.26 (1H, m), 6.70-6.62 (2H, m), 3.85 (3H, s, $COOCH_3$).
(Sold by Fluka Co., mp 21°C).

H. AI-15



0.75 g, 5 mM, AG 949 and 0.7 g, 5 mM, gentise aldehyde in 30 ml methanol were refluxed 1 hour. The red solution was cooled and 0.5 g $NaCNBH_4$ was added. After 3

hours at room temperature it was extracted with EtAc. Evaporation and trituration with benzene gave 0.25 g, 18% yield, of light yellow solid, mp 175°C.

MS 274 (10%), 273 (M^+, 61%), 240 (12), 152 (27), 151 (M-$C_7H_5(OH)_2$, 100%), 120 (42), 119 (98), m/e.

NMR acetone-d_6 δ 7.86 (1H, m, dd), 7.34 (1H, m, dt), 6.73 (3H, m), 6.56 (2H, m), 4.42 (2H, s, CH_2N), 3.83 (3H, s, OCH_3).

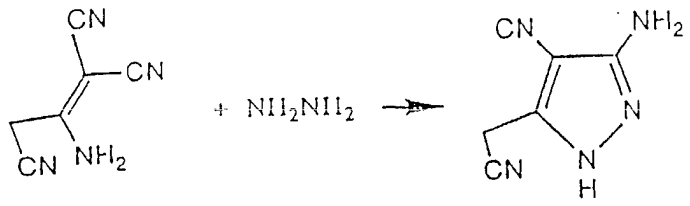
I. AI-16

0.7 g, 5.1 mM, 2,5-dihydroxy benzaldehyde and 0.75 g, 5.0 mM 3-amino methyl benzoate in 40 ml methanol were refluxed 3 hours, cooled, and 0.5 g $NaCNBH_4$ were added. After 12 hours at room temperature workup (H_2O, EtAc) and chromatography (silica gel, elution with 5% CH_3OH in CH_2Cl2) gave 0.42, 31% yield, light yellow solid, mp 175°C. NMR acetone-d_6 δ 7.78, 6.68 (4H, ABq, J_{AB}=8.8 Hz), 6.74 (1H, d, J=3.0 Hz,H_6), 6.72 (1H, d, J=8.5 Hz,H_3), 6.55 (1H, d, J=8.5, 3.0 Hz,H_4), 4.34 (2H, s, CH_2N), 3.76 (3H, s, $COOCH_3$).

Example 5

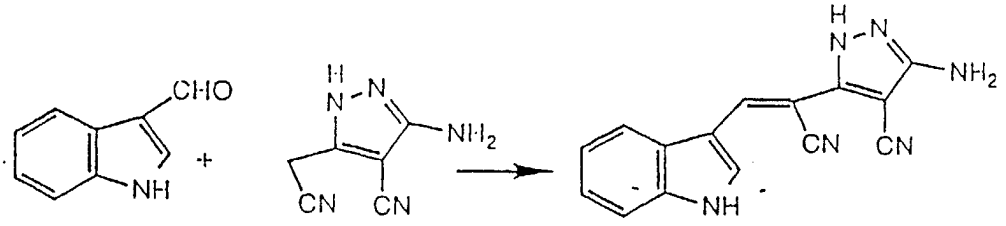
Preparation of Group II compounds.

A. AG567



The compound was prepared according to Carboni *et al*. 2.2 g malononitril dimer and 0.9 ml N_2H_4 in 20 ml water were heated 15 minutes at 100°C. Cooling and filtering gave 1.5 g; 61% yield, white solid, mp 187°C (Carboni et al., mp 187°C). NMR acetone d_6 δ3.88(s).

B. AII-20



0.29 g, 2 mM, 3-formyl indole, 0.29, 2 mM, AG 567 and 20 mg β-alanine in 30 ml ethanol were refluxed 4 hours. Cooling and filtering gave 0.34 g, 62% yield, yellow solid, mp. 281°C.

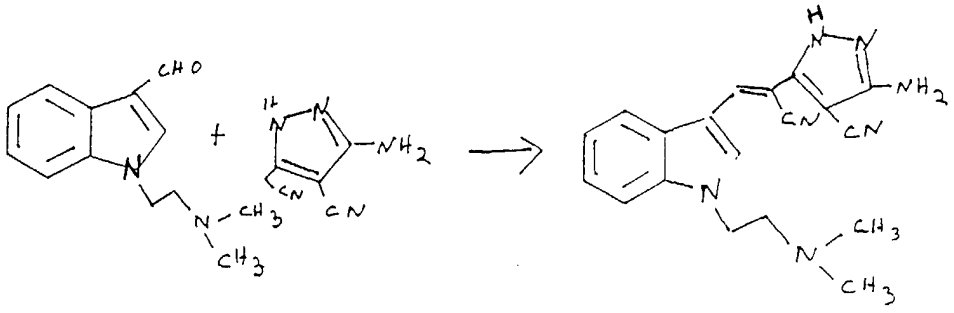
NMR acetone $d_6\delta$ 8.52 (1h,S, Vinyl), 8.42 (1H,S,H_2), 7.79 (1H,m), 7.75 (1H,m), 7.27 (2H,m), 6.17 (1H, Br.S, NH), MS-274 (M^+, 100%), 219(14), 91(35), m/e.

C. AII-21

0.3 g (1.3 mM) 3-amino-4-cyano-5-cyanomethyl-2-pyrazole, 0.2 g (1.36 mM) of 1-(3-dimethylaminopropyl)-3-formyl indole and 20 mg β-alanine in 20 ml ethanol were refluxed 4 hours. Evaporation, trituration with benzene and filtering gave 0.4 g of yellow solid (94% yield) containing 10% 3-amino-4-cyano-5-cyanomethyl-2-pyrazole. 0.4 g was chromatographed on silica gel (70-220 mesh) eluting with 85:15 methylene chloride:methanol to give 0.12 g of a bright yellow solid having a melting point of 250°C.

NMR acetone $d_6\delta$ 8.45($1H_1S_1$vinyl), 8.37($1H_1S_1H_2$), 7.78($1H_1$m), 7.60($1H_1$m). 7.28($2H_1$m), 4.47($2H_1t_1J$=6.8H_z), 2.29($2H_1t_1J$=6.8H_2), 2.24(6H,S,N-)$CH_3)_2$). MS-360(M+1, 8%), 359(M+,31), 289(100), 261(15), 144(6), m/e.

D. AII-22



0.6 g, 2.8 mM, 0.4 g, 2.7 mM, AG 567 and 20 mg β-alanine in 25 ml ethanol were refluxed 4 hours. Evaporation and chromatography gave 0.12 g, 13% yield, yellow solid, mp-252°C.

NMR acetone d_6 δ 8.52(1H,S,Vinyl), 8.37(1H,S,H_2), 7.80(1H,m), 7.60(1H,m), 7.30(2H,m), 4.49(2H,t,J=6.6H_z), 2.79(2H,t,J=6.6H_z), 2.28(6H,S).
MS-345(M+,100%), 198(55%), 147(M-198, 25), 117(45), m/e.

Example 6

Preparation of Group III Compounds.

A. AIII-30

AIII-30 preparation is described by Gazit *et al., J. Med. Chem. 32*:2344 (1989).

B. AIII-31

AIII-31 preparation is described by Gazit *et al, J. Med. Chem:34*, 1896 (1991).

C. AIII-32

AIII-32 preparation is described by Gazit *et al.*, *J. Med. Chem. 34*:1896 (1991).

D. AIII-33

AIII-33 preparation is described by Gazit *et al., J Med. Chem. 34*:1896 (1991).

E. AIII-34

AIII-34 was synthesized using a two step procedure.

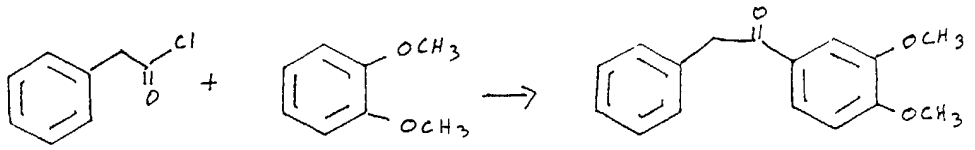
(i). Synthesis of 3-amino-4-cyano-5-cyanomethyl-2 pyrozole:

2.2 g malononitrile dimer and 0.9 ml N_2H_4 in 20 ml of water were heated for 15 minutes at 100°C. Cooling and filtering gave 1.5 g (61% yield) of a white solid having a melting point of 187°C. (NMR acetone d_6 δ 3.88 (s).) (Cf. Carboni *et al., J. Am. Chem. Soc. 80*:2838 (1958), reporting m.p. 197°C.

(ii). Condensation with dihydroxybenzaldehyde:

To 0.28 g (2 mM), 3,4-dihydroxybenzaldehyde and 1.33 g (2.2 mM of 3-amino-4-cyano-5-cyanomethyl-2 pyrozole in 20 ml ethanol were added three drops piperidine and the reaction was refluxed 3 hours. Cooling, filtering and washing with ethanol gave 1.3 g (56% yield) of a yellow solid having a melting point of 300°C.

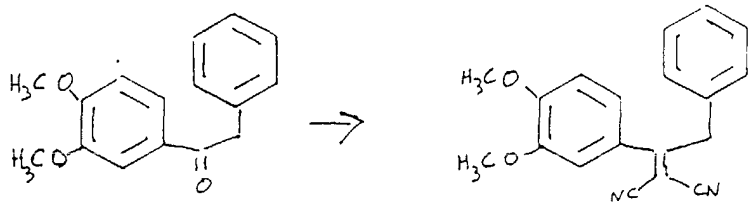
F. AG 604



To 6.3 ml, 50 mM, veratrole and 7 ml, 53 mM, phenyl acetyl chloride in 50 ml CH_2Cl_2 was added 6.7 g $AlCl_3$. After 1.5 hours stirring at room temperature the violet colored reaction was decomposed and extracted with CH_2Cl_2 to give a red oil. Trituration with ethanol-hexane, filtering and washing with hexane gave white solid, 8 g, mp 72°C, yield 62%.

NMR $CDCl_3$ δ 7.66 (2H, m), 7.54 (1H, d, J=2.0 Hz, H_2), 7.40-7.20 (4H, m), 6.87 (1H, d, J=8.4 Hz, H_5), 4.24 (2H, s, CH_2), 3.93, 3.90 (6H, 2s, OCH_3).

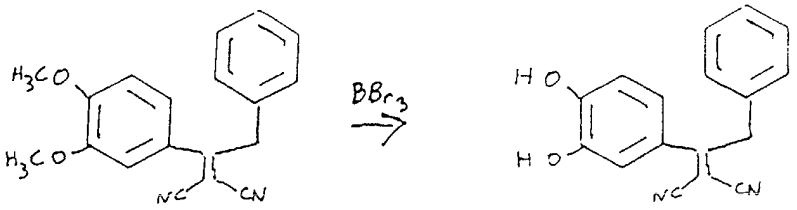
G. AG 660



To 5 g, 20 mM, AG 604 and 2 g, 30 mM, malononitrile in 30 ml ethanol was added 0.4 g β-alanine. The reaction was refluxed 65 hours, evaporated and chromatographed on silica gel. The yellow band was collected and recrystallized from ethanol to give 3.5 g, 54% yield of yellow solid, mp 123°C.

NMR $CDCl_3$ δ 7.30-7.10 (6H, m), 7.01 (1H, d, J=2.2 Hz, H_2), 6.90 (1H, d, J=8.4 Hz, H_5), 4.26 (2H, s, CH_2), 3.93, 3.85 (6H, s, OCH_3).

H. AIII-35

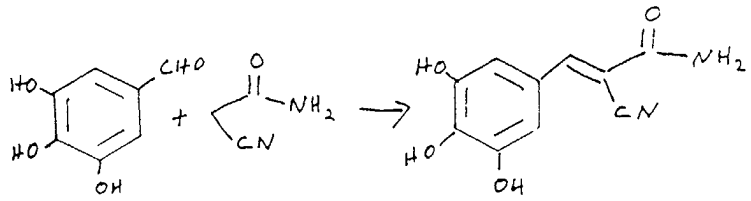


To 0.64 g, 2.1 mM, AG 660 in 20 ml CH_2CI_2, under argon, was added 1.2 ml, 12 mM, BBr_3 and the reaction stirred 1.5 hours at room temperature. The color after addition is red, turns green, and then after 1 hour light red. Water was added and the reaction extracted with EtAc. Evaporation gave solid which was triturated with CH_2Cl_2 and filtered to give 0.52 g, 90% yield, of yellow-green solid, mp 260°C.

NMR acetone-d_6 δ 7.61-7.20 (7H, m), 6.95 (1H, d, J=8.6 Hz, H_5), 4.36 (2H, s, CH_2).

MS - 276 (M^+, 100%), 262 (M-14, 14%), 137 (99%), m/e.

I. AIII-36



To 0.5 g, 3 mM Gallic aldehyde and 0.27, 3.1 mM, Cyano acetamide in 3 ml ethanol was added 2 drops piperidine and the reaction refluxed 3 hours. Cooling, washing with ethanol and drying gave 0.54, 78% yield, yellow-orange solid, mp-295°C.

NMR acetone d_6 δ 7.97 (1H, S, vinyl), 7.18 (2H, S, $H_{2.6}$).

J. AIII-37

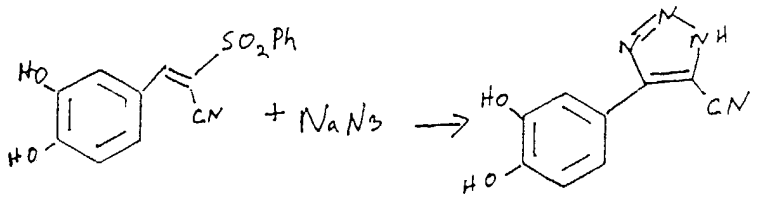
340 mg (1.5 mM) 1-phenyl-3-amino-4-cyano-5-cyanomethyl-2-pyrazole, 210 mg (1.5 mM) 3,4-dihydroxy benzaldehyde and 4 drops of piperidine in 30 ml ethanol were refluxed for 6 hours. Cooling and filtering gave 145 mg yellow solid. Evaporation of the solvent and trituration with CH_2Cl2-acetone gave another 145 mg yellow solid (56% yield). The product had a melting point of 147°C.

NMR acetone d_6 δ-7.87 (1H,S, Vinyl), 7.68 (1H,d, J=2.2 H_z, H_2) 7.66-7.45 (5H,m, Ph), 7.28 (1H,dd, J=8.3.2.2 H_z, H_6). 6.92 (1H,d,J=8.3 H_z, H_5).

Example 7

Preparation of Group VI (other compounds).

A. AIV-40

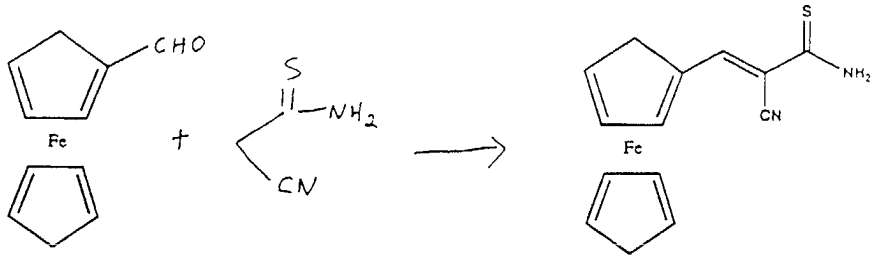


To 0.9 g, 3 mM, of the above sulphonyl cyano compound in 3 ml DMF was added 0.3 g, 3 mM NaN_3. The reaction was heated 3 hours at 100°C, water and HCl added, and the reaction mixture was extracted with EtAc. Evaporation gave a solid which was triturated with CH_2Cl_2 and filtered to give 0.44 g, 73% yield, white solid, mp 283°C.

MS 202 (M^+, 100%), 164 (45%), 163 (63%), 147 (M-N_2-HCN, 9%), m/e.

NMR acetone-d_6 δ 7.48 (1H, d, J=1.8 Hz, H_2), 7.40 (1H, dd, J=8.2, 1.8 Hz, H_6), 7.02 (1H, d, J=8.2 Hz, H_5).

B. AIV-41

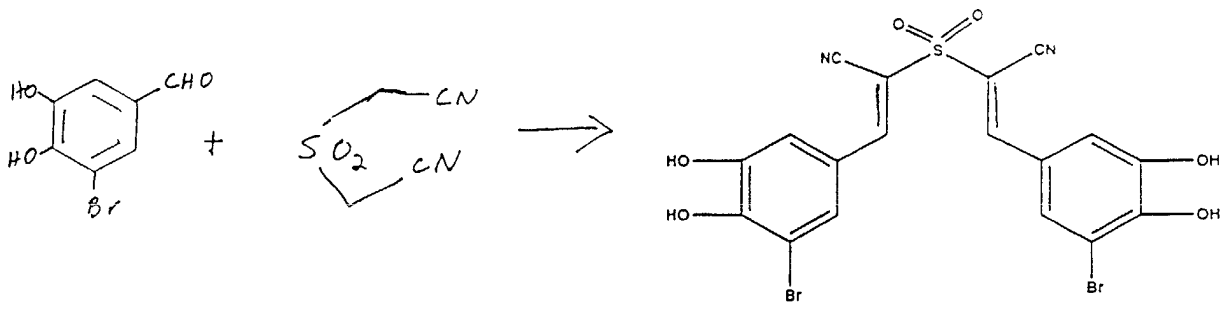


To 0.6 g, 3 mM, ferrocene aldehyde and 0.3 g, 3 mM, thiocyano acetamide in 10 ml ethanol were added 3 drops piperidine. The reaction was refluxed 2.5 hours, water and HCl added, and the reaction extracted with CH_2Cl_2. Evaporation gave 0.6 g violet solid, 72% yield, mp. 182°C.

MS-297 (M+1, 18%), 296 (M+, 100%), 270 (12), 262 (30), 233 (17), 231 (M-C_5H_5, 40), 230 (24), 220 (33), 219 (19), 199 (63), 197 (52), 186 (15), 155 (29), 146 (15), 137 (14), 121 (C_5H_5Fe+, 81) m/e.

NMR $CDCl_3$ δ 8.75 (1H, S, vinyl), 5.06 (2H, t, J=1.9 H_2, $H_{2,5}$) 4.82 (2H, t, J=1.9 H_2, $H_{3,4}$), 4.32 (5H,S).

C. AIV-42



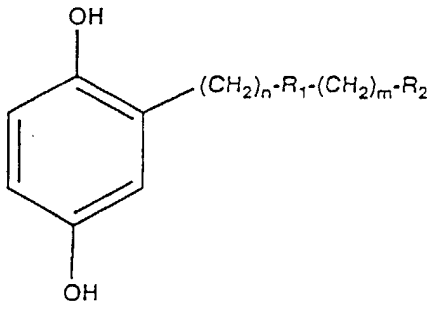
230 mg, 1.06 mM, 3,4-hydroxy 5-bromo benzaldehyde, 76 mg, 0.53 mM, diacetonitrile sulphone and 10 mg β-alanine in 10 ml ethanol were refluxed 5 hours. Cooling and filtering gave 220 mg, 76% yield, orange solid, mp > 300°C. NMR acetone d_6 δ 8.18(2H,S, vinyl), 7.90 (2H, d,J=1.6 Hz), 7.78(2H,d,J=1.6 Hz).

Other embodiments are within the following claims.

CLAIMS

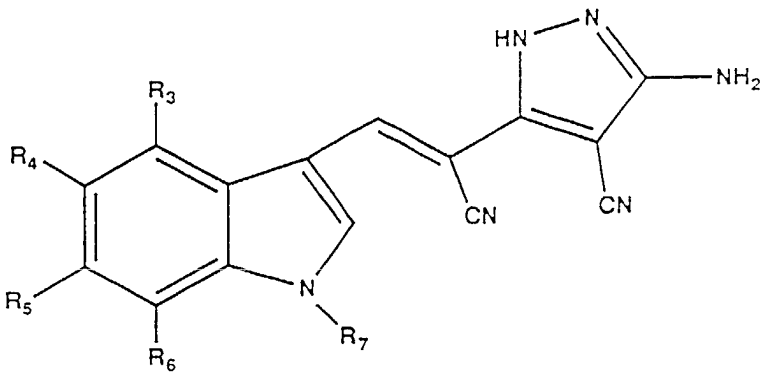
1. An agent for treating a patient having a cell proliferative disorder characterized by abnormal *abl* activity comprising a compound selected from the group consisting of:

a) a compound of the chemical formula:

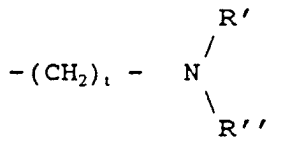


wherein R_1 is selected from the group consisting of NH, O, and S; R_2 is substituted phenyl having 1 to 3 substituents independently selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino, n is an integer between 0 and 6, and m is an integer between 0 and 6, provided that if n is 1 and m is 0, then said substituted phenyl is not 2-$CO(NH_2)$-phenyl or 4-$(COOCH_3)$-phenyl;

b) a compound of the chemical formula:

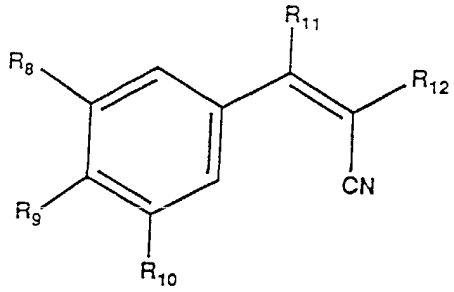


wherein R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2, and NH_2; and R_7 is selected from the group consisting of H and:



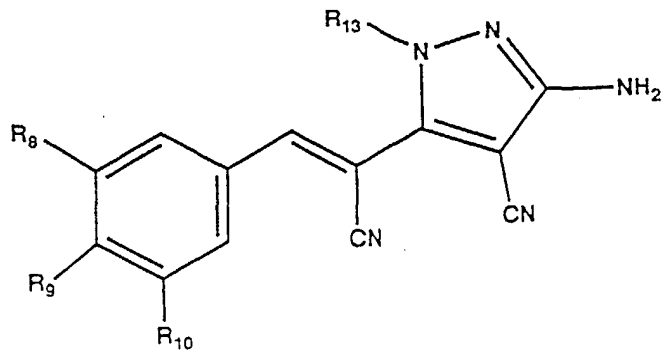
wherein t is an integer between 1 and 12, and R′ and R′′ is each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, alkoxy and aryl;

c) a compound of the chemical formula:



wherein R_8, R_9, and R_{10}, is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is an alkylaryl; and R_{12} is selected from the group consisting of aryl, further substituted aryl, CN, amide, and thioamide,

d) a compound of the chemical formula:



wherein R_8, R_9, and R_{10} is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen hydrogen, NO_2 and NH_2;

and R_{13} is substituted phenyl having 1 to 3 substituents independently selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino, or phenyl;

d) AIV-41; and

e) AIV-42;

wherein said compound inhibits growth of a cell having abnormal *abl* activity.

2. The agent of claim 1, wherein said disorder is a leukemia.

3. The agent of claim 2, wherein said disorder is chronic myelogenous leukemia.

4. The agent of claim 2, wherein said disorder is acute lymphoblastic leukemia.

5. The agent of claim 2, wherein said compound inhibits activity of a *bcr-abl* fusion protein.

6. The agent of claim 5, wherein said *bcr-abl* fusion protein is p210 *bcr-abl*.

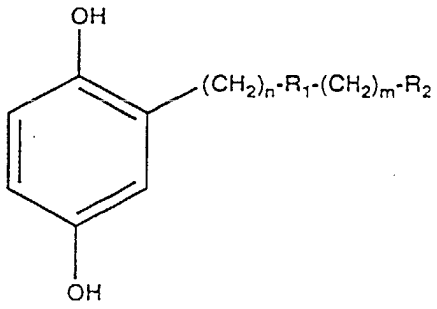
7. The agent of claim 5, wherein said *bcr-abl* fusion protein is p185 *bcr-abl*.

8. A compound selected from the group of compounds consisting of AI-10, AI-11, AI-12, AI-14, AI-15, AII-20, AII-21, AII-22, AIII-35, AIII-37, AIV-41, and AIV-42.

9. The compound of claim 8, selected from the group consisting of AI-10, AI-11, AI-12, AI-14, AI-15, and AII-20.

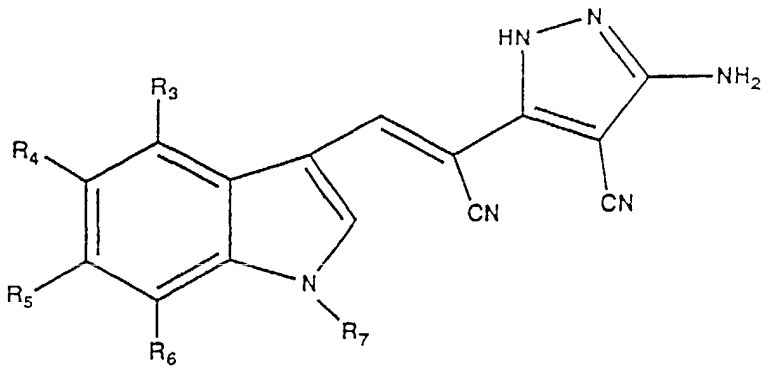
10. A composition comprising a therapeutically effective amount of a compound selected from the group consisting of:

a) a compound of the chemical formula:

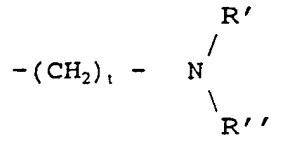


wherein R_1 is selected from the group consisting of NH, O, and S; R_2 is substituted phenyl having 1 to 3 substituents independently selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino, n is an integer between 0 and 6, and m is an integer between 0 and 6, provided that if n is 1 and m is 0, then said substituted phenyl is not 2-$CO(NH_2)$-phenyl or 4-$(COOCH_3)$-phenyl;

b) a compound of the chemical formula:

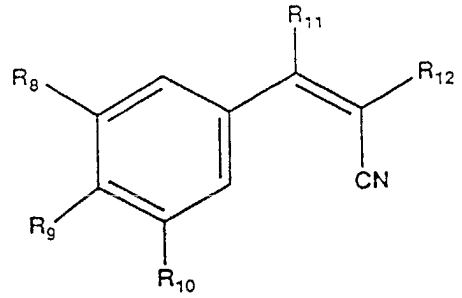


wherein R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2, and NH_2; and R_7 is selected from the group consisting of H and:



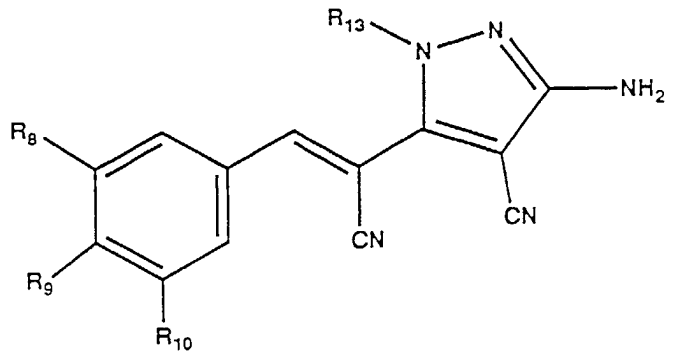
wherein t is an integer between 1 and 12, and R′ and R′′ is each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, alkoxy and aryl;

c) a compound of the chemical formula:



wherein R_8, R_9, and R_{10}, is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is an alkylaryl; and R_{12} is selected from the group consisting of aryl, further substituted aryl, CN, amide, and thioamide,

d) a compound of the chemical formula:



wherein R_8, R_9, and R_{10} is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen hydrogen, NO_2 and NH_2;

and R_{13} is substituted phenyl having 1 to 3 substituents independently selected from the group consisting of ester, amide, thioamide, thioether, halogen, trihalomethyl, OH, SH, NO_2, alkoxy, cyano, and amino, or phenyl;

d) AIV-41; and

e) AIV-42; and

a pharmacologically acceptable carrier; wherein said compound inhibits growth a cell having abnormal *abl* activity.

11. The composition of claim 10, wherein said disorder is a leukemia.

12. The composition of claim 11, wherein said disorder is chronic myelogenous leukemia.

13. The composition of claim 10, wherein said disorder is acute lymphoblastic leukemia.

14. The composition of claim 11, wherein said compound inhibits activity of a *bcr-abl* fusion protein.

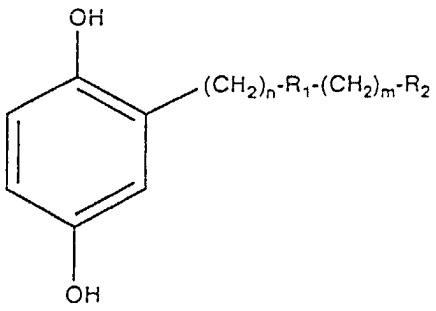
15. The composition of claim 14, wherein said *bcr-abl* fusion protein is p210 *bcr-abl*.

16. The composition of claim 14, wherein said *bcr-abl* fusion protein is p185 *bcr-abl*.

17. A method of treating a patient having a cell proliferative disorder characterized by abnormal *abl* activity comprising the step of administering to said patient a therapeutically effective amount of a compound which inhibits said *abl* activity *in vivo*.

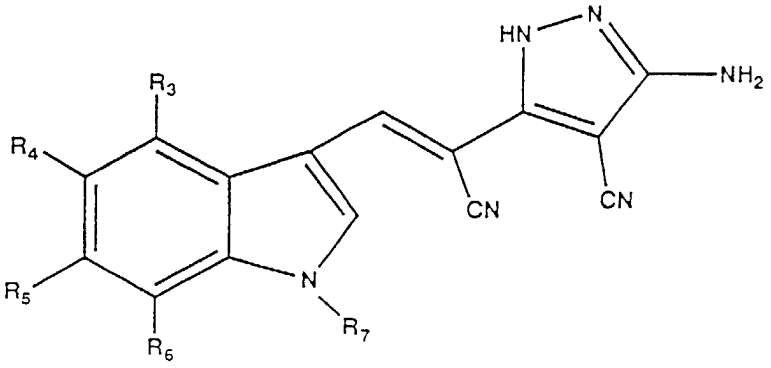
18. A method of treating a patient having a cell proliferative disorder characterized by abnormal *abl* activity comprising the step of administering to said patient a therapeutically effective amount of a compound selected from the group consisting of

a) a compound of the chemical formula:



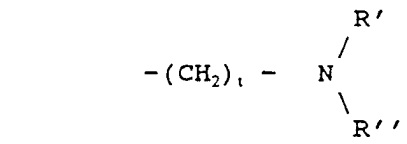
wherein R_1 is selected from the group consisting of NH, O, and S; R_2 is an aryl, n is an integer between 0 and 6, and m is an integer between 0 and 6;

b) a compound of the chemical formula:



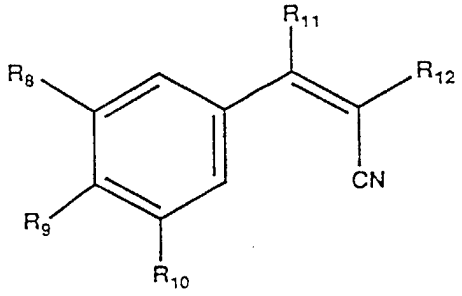
wherein R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of alkyl, alkenyl, alkynyl,

alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2, and NH_2; and R_7 is either H or has the chemical formula:



wherein t is an integer between 1 and 12, and R′ and R′′ is each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, alkoxy and aryl;

c) a compound of the chemical formula:



wherein R_8, R_9, and R_{10}, is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is selected from the group consisting of an H, alkyl, and alkylaryl; and R_{12} is selected from the group consisting of aryl, further substituted aryl, CN, amide, and thioamide,

c) AIV-40;

d) AIV-41; and

e) AIV-42;

wherein said compound inhibits growth of a cell having abnormal *abl* activity.

19. The method of claim 20, wherein said disorder is a leukemia.

20. The method of claim 19, wherein said disorder is chronic myelogenous leukemia.

21. The method of claim 19, wherein said disorder is acute lymphoblastic leukemia.

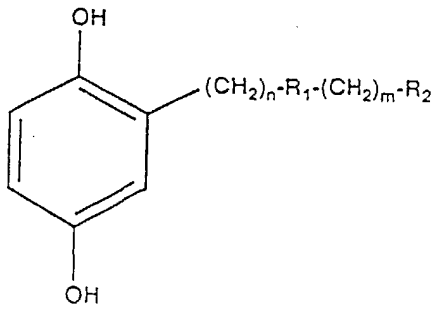
22. The method of claim 19, wherein said compound inhibits activity of a *bcr-abl* fusion protein.

23. The method of claim 22, wherein said *bcr-abl* fusion protein is p210 *bcr-abl*.

24. The method of claim 22, wherein said *bcr-abl* fusion protein is p185 *bcr-abl*.

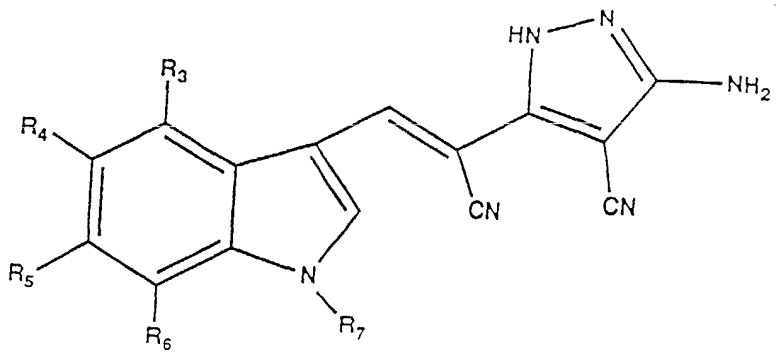
25. A method of inhibiting or decreasing proliferation of cells having enhanced proliferation due to abnormal *abl* activity which comprises exposing said cells to a cell proliferation decreasing effective amount of a compound selected from the group consisting of:

a) a compound of the chemical formula:

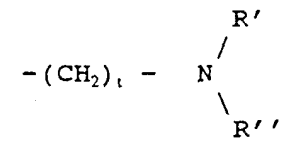


wherein R_1 is selected from the group consisting of NH, O, and S; R_2 is an aryl, n is an integer between 0 and 6, and m is an integer between 0 and 6;

b) a compound of the chemical formula:

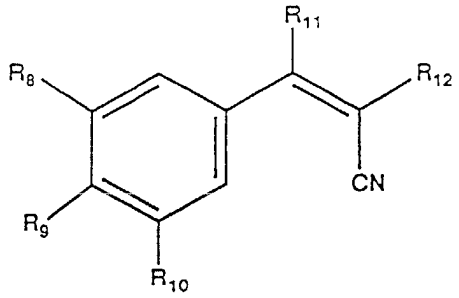


wherein R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2, and NH_2; and R_7 is either H or has the chemical formula:



wherein t is an integer between 1 and 12, and R′ and R′′ is each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, alkoxy and aryl;

c) a compound of the chemical formula:



wherein R_8, R_9, and R_{10}, is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is selected from the group consisting of an H, alkyl, and alkylaryl; and R_{12} is selected from the group consisting of aryl, further substituted aryl, CN, amide, and thioamide,

c) AIV-40;

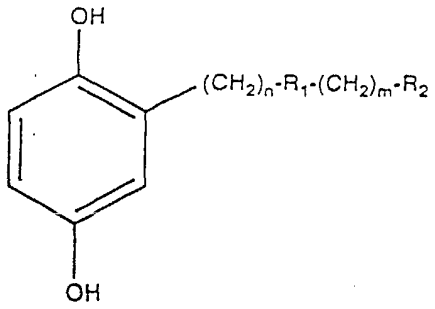
d) AIV-41; and

e) AIV-42;

wherein cell proliferation is decreased.

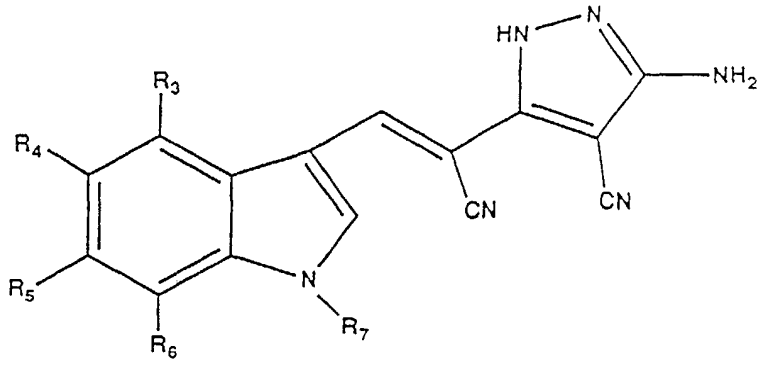
26. A method of inhibiting or decreasing proliferation of cells having enhanced proliferation due to abnormal *abl* activity which comprises exposing said cells to an amount effective to inhibit or decrease activity of a *bcr-abl* fusion protein selected from the group consisting of:

a) a compound of the chemical formula:



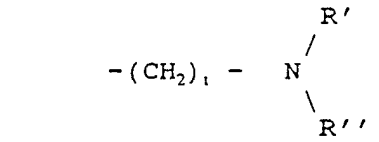
wherein R_1 is selected from the group consisting of NH, O, and S; R_2 is an aryl, n is an integer between 0 and 6, and m is an integer between 0 and 6;

b) a compound of the chemical formula:



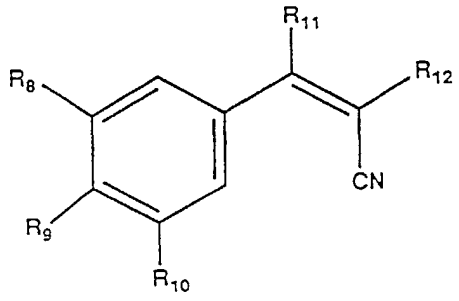
wherein R_3, R_4, R_5, and R_6 is each independently selected from the group consisting of alkyl, alkenyl, alkynyl,

alkoxy, alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2, and NH_2; and R_7 is either H or has the chemical formula:



wherein t is an integer between 1 and 12, and R′ and R′′ is each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, alkoxy and aryl;

c) a compound of the chemical formula:



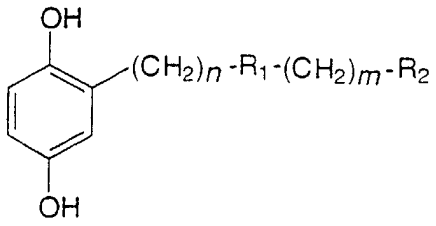
wherein R_8, R_9, and R_{10}, is each independently selected from the group consisting of alkyl, alkenyl, alkynyl, alkoxy alkylaryl, OH, amine, SH, halogen, hydrogen, NO_2 and NH_2; R_{11} is selected from the group consisting of an H, alkyl, and alkylaryl; and R_{12} is selected from the group consisting of aryl, further substituted aryl, CN, amide, and thioamide,

c) AIV-40;

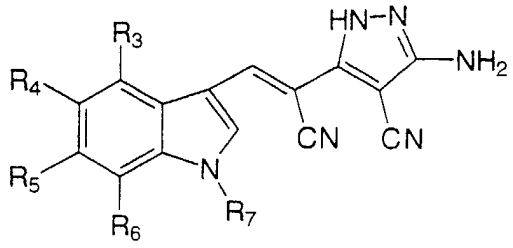
d) AIV-41; and

e) AIV-42;

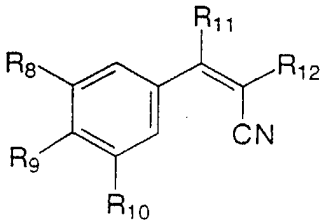
wherein cell proliferation is decreased.



GROUP I

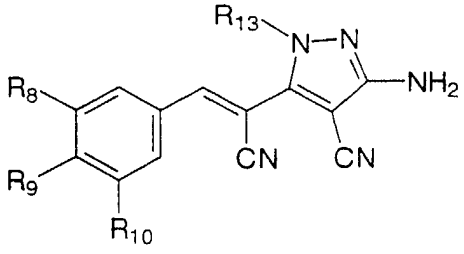


GROUP II



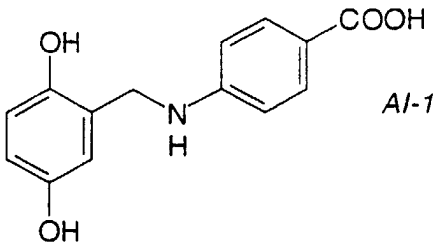
GROUP III

FIG. 1

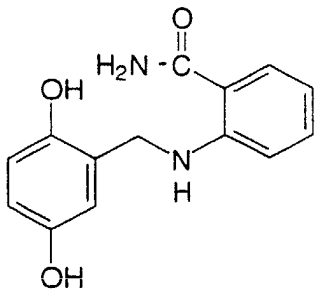


GROUP IIIA

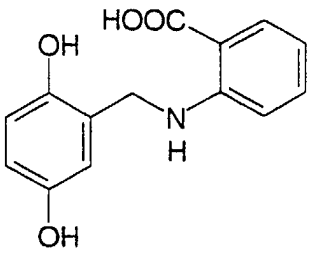
FIG. 2



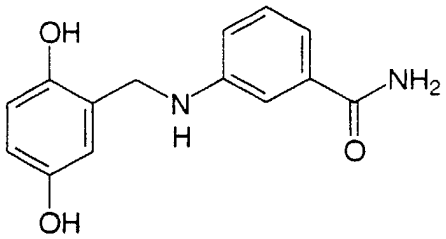
AI-10



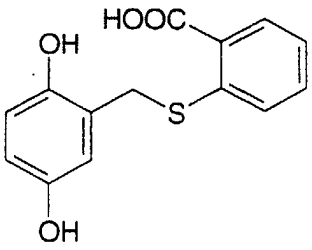
AI-13



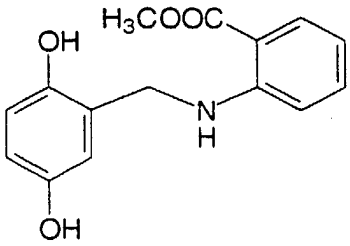
AI-11



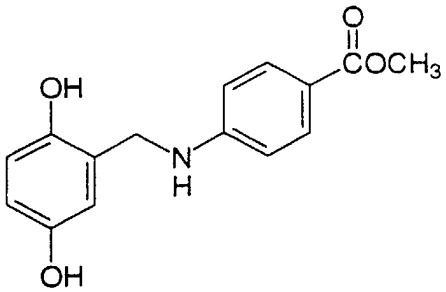
AI-14



AI-12



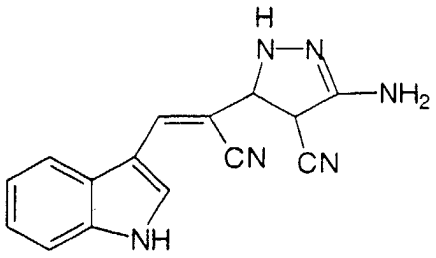
AI-15



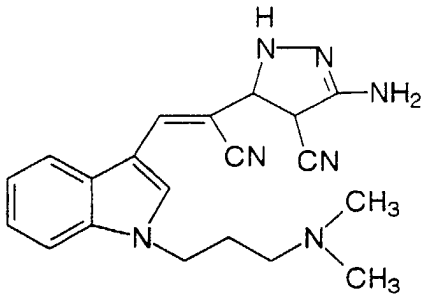
AI-16

FIG. 3A

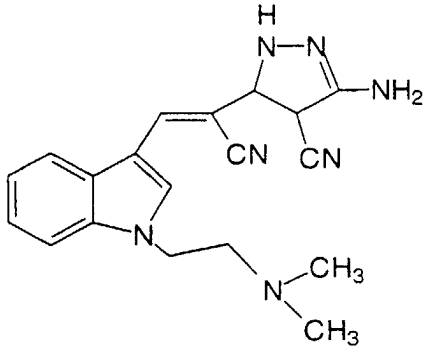
GROUP I



AII-20



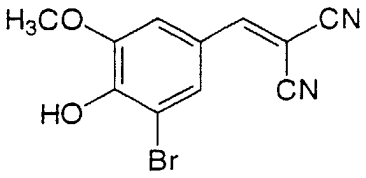
AII-21



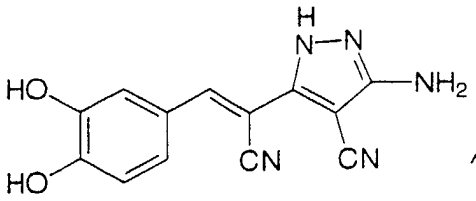
AII-22

FIG. 3B

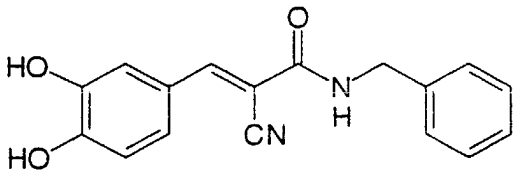
GROUP II



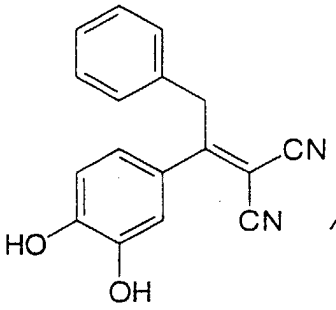
AIII-30



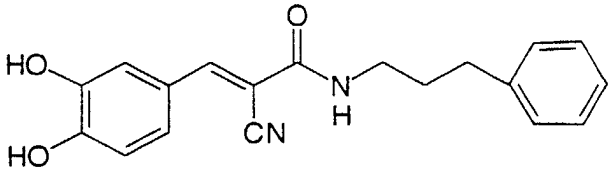
AIII-34



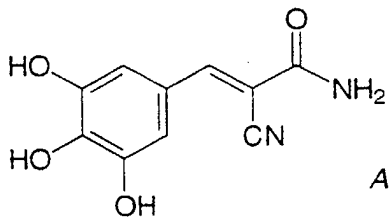
AIII-31



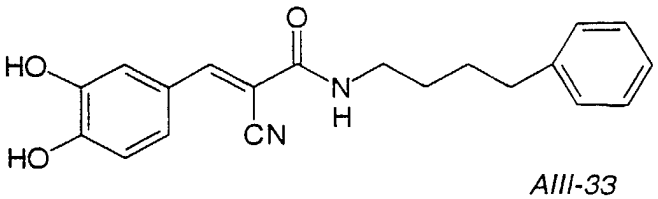
AIII-35



AIII-32



AIII-36



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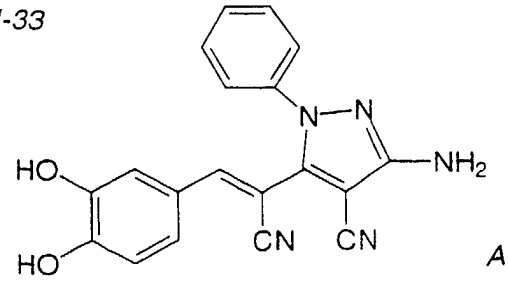
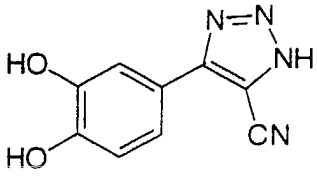
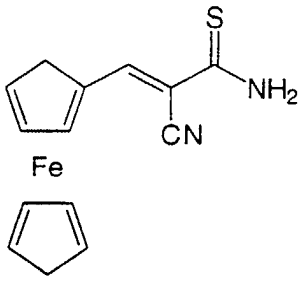


AIII-37

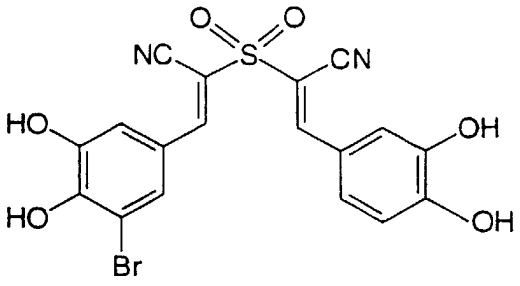
FIG. 3C
GROUP III



AIV - 40



AIV - 41



AIV - 42

FIG. 3D
GROUP IV (OTHERS)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/05294

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K31/165 A61K31/24 A61K31/195 A61K31/275 A61K31/415

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J.BIOL.CHEM., vol.267, no.7, 1992 pages 4518 - 23 'Selective interactions of transforming and normal abl proteins with ATP, tyrosine-copolymer substrates, and tyrphostins' see page 4518, left column, line 1 - right column, line 6 see page 4519, right column, line 6 - line 30 see page 4520, table I, compounds AG957, AG952 see page 4522, right column, line 19 - line 23 --- -/--	1-26

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 1994	10. 10. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Gerli, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/05294

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TRENDS IN PHARMACOLOGICAL SCIENCES, vol.12, 1991 pages 171 - 3 'Tyrphostins as molecular tools and potential antiproliferative drugs' see page 171, column 2, line 26 - column 3, line 18 see page 172; table I ---	1-26
Y	FASEB J., vol.6, no.14, 1992 pages 3257 - 82 'Tyrphostins: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction' see page 3279, right column, line 26 - line 30 see page 3279, line 59 - line 61 see page 3280; table I ---	1-26
Y	J.MED.CHEM., vol.32, 1989 pages 2344 - 52 'Tyrphostins I: Synthesis and biological activity of protein tyrosine kinase inhibitors' see page 2347; table III -----	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05294

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 17-26 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compounds/composition.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.